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STUDIES ON THE ISOLATED ISLET TISSUE OF FISH.
III. THE EFFECT OF SUBSTRATES AND INHIBITORS ON THE OXYGEN UPTAKE OF PANCREATIC ISLET SLICES OF TOADFISH [1]

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Little is known about the metabolic activity of islet tissue because in mammalian species this tissue is dispersed into a million or more individual islets of Langerhans, whose total mass approximates only 1% of the pancreatic mass. This makes it very difficult to separate the islet from the acinar tissue. In teleost fish, however, the islet tissue is concentrated into one or more discrete bodies called the principal islets (Diamare, 1899); the acinar tissue, in contrast to that of mammalian species, is dispersed throughout the mesentery and located along the bile duct and within the liver. The toadfish (*Opsanus tau*) was used for this metabolic study of islet tissue because of the accessibility of the islets and the general availability and hardiness of this species.

In a previous study (Lazarow, Cooperstein, Bloomfield and Friz, 1957), the oxygen uptake of islet tissue was measured under varying conditions of pH, tonicity, and electrolyte composition, and the baseline conditions under which maximal respiration of islet tissue occurs were defined. The present paper reports further characterization of the over-all metabolic pathways of islet tissue achieved by studying the effects of specific exogenous substrates and inhibitors on the respiration of islet slices.

Material and Methods

After the toadfish were killed by a blow on the head, the principal islets were dissected from the mesentery and the capsules of the islets were removed. The latter is an important step in the procedure, since any acinar tissue that may be present underneath the capsule, as well as any acinar tissue that may be present

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within the islet but attached to the capsule by trabeculae, is removed when the capsule is stripped.

In each experiment a single islet was cut into eight slices and these slices were distributed between the experimental and control groups. The Cartesian diver microrespirometer was used to measure the oxygen uptake of each toadfish slice, in a manner previously described (Lazarow *et al.*, 1957). A modification (Lazarow *et al.*, 1957) of the method of Lowry, Rosebrough, Farr and Randall (1951) was used to measure the protein content of each slice. The metabolic activity was expressed as millimicroliters (mμl.) of oxygen per microgram (μg.) of protein per hour.

The metabolic activity of toadfish liver tissue was also studied in order to compare it with that of toadfish islet tissue. The Warburg manometer was used to make these measurements, and the oxygen uptakes were also expressed in millimicroliters per microgram of protein per hour.

A constant amount of phosphate buffer at pH 7.4 (0.054 *M*) was present in all of the experiments in this study. The respiration of islet tissue was studied in a medium containing phosphate plus substrate or inhibitor, and the oxygen uptakes thus obtained were compared to those observed in either a medium containing phosphate plus saline or a medium containing phosphate alone.[3]

The various solutions of substrates and inhibitors were prepared at twice the desired final concentration and mixed with an equal volume of 0.108 *M* phosphate buffer so that the final phosphate concentration was 0.054 *M*.

Results

Effect of substrates

The substrates studied included glucose, pyruvate (lithium salt), α-ketoglutarate, glutamate, succinate, and isocitrate.

When glucose was added to the medium *in vitro* (Table I) neither islet ($p = 0.21$) nor liver ($p = 0.9$) respiration was altered. It should be noted that the endogenous metabolic activity of liver tissue slices was considerably higher than that of islet tissue.

In a similar series of experiments using pyruvate as the substrate, respiration of islet slices was compared in the following media: (a) a hypotonic medium containing phosphate; (b) an isotonic medium containing phosphate plus NaCl; and (c) a medium containing phosphate plus pyruvate (Table II). If one assumes that the added pyruvate readily enters the islet cells, then the respiration of islet slices in the pyruvate-containing medium should be compared with that in the hypotonic medium because (a) and (c) would be of equal tonicity. When these media are in fact compared, it is noted that pyruvate has no effect on the respiration of islet tissue. If, on the other hand, one assumes that the added pyruvate does *not* enter the islet cells, then media (b) and (c) should be compared since

[3] Whereas Lazarow *et al.* (1957) reported that optimal respiration of islet slices was observed in a hypotonic medium (0.054 *M* phosphate), in the present study optimal respiration of islet slices was sometimes observed in an isotonic medium whereas at other times no difference was noted between respiration in hypotonic and isotonic media. Evidence indicates that part of this discrepancy may be due to the varying temperature of the water in which the fish were kept. Further work is now in progress in order to clarify these differences.

TABLE I

Effect of glucose on the respiration of toadfish islet and liver slices

Medium	Tonicity of medium (equiv. NaCl conc.* *M*/liter)	Islet			Liver		
		No. of deter.	Ave. mμl. O_2/μg. prot./hr.	σ†	No. of deter.	Ave. mμl. O_2/μg. prot./hr.	σ†
0.054 *M* PO_4	0.0755	17	1.5	0.34	8	2.8	0.91
0.054 *M* PO_4 + 0.0645 *M* NaCl	0.140	12	1.9	0.11	8	2.8	0.71
0.054 *M* PO_4 + 0.129 *M* glucose	0.140	11	1.9	0.98	9	2.7	0.97

* The concentration of a sodium chloride solution whose tonicity would equal that of the medium used.

† σ = Standard Deviation. The statistical significance of the results (p) was calculated using the following equation:

$$p = \frac{\text{difference}}{\sqrt{\frac{(\sigma_1)^2}{N_1 - 1} + \frac{(\sigma_2)^2}{N_2 - 1}}}$$

where σ_1 and σ_2 are the standard deviations of the control (without substrate) and experimental (with substrate) groups, respectively; N_1 and N_2 are the corresponding number of determinations in each group.

they would be of approximately equal tonicity. Comparison of the respiration in *these* media indicates that pyruvate addition inhibits by 23% ($p = 0.019$).

The addition of α-ketoglutarate (Table III) increased the oxygen uptake of islet slices by approximately 33% when compared with either a hypotonic ($p = 0.07$) or isotonic ($p = .008$) medium.

Table IV shows that a 44% increase in oxygen uptake was observed when islet slices were placed in a phosphate-glutamate medium ($p = 0.016$). This is to be compared to a 30–36% increase observed when toadfish liver slices were placed in the same medium ($p = <0.001$).

Oxygen uptake was also increased when succinate (Table V) was added either to toadfish islet or to toadfish liver tissue. The increase in islet tissue respiration in an isotonic phosphate-succinate medium was 47% ($p = 0.01$) when compared to respiration in an isotonic phosphate-saline medium and 100% ($p = 0.001$) when

TABLE II

Effect of pyruvate on the respiration of toadfish islet slices

Series	Medium	Tonicity of medium (equiv. NaCl conc. *M*/liter)	No. of deter.	Ave. mμl. O_2/μg. prot./hr.	σ
a	0.054 *M* PO_4	0.0755	43	1.7	0.70
b	0.054 *M* PO_4 + 0.0645 *M* NaCl	0.140	14	2.2	0.69
c	0.054 *M* PO_4 + 0.040–0.0645 *M* pyruvate	0.1155–0.140	28	1.7	0.70

TABLE III

Effect of α-ketoglutarate on the respiration of toadfish islet slices

Medium	Tonicity of medium (equiv. NaCl conc. *M*/liter)	No. of deter.	Ave. mμl. O_2/μg. prot./hr.	σ
0.054 *M* PO_4	0.0755	13	2.1	0.83
0.054 *M* PO_4 + 0.0645 *M* NaCl	0.140	13	2.0	0.60
0.054 *M* PO_4 + 0.0645 *M* α-ketoglutarate	0.140	14	2.7	0.84

compared to respiration in the hypotonic medium. The addition of succinate increased the respiration of toadfish liver slices by about 75% ($p = <0.001$).

In contrast to α-ketoglutarate, glutamate, and succinate, another Krebs cycle intermediate, isocitrate, did not stimulate islet respiration (Table VI).

Effect of inhibitors

The use of inhibitors on whole cells, extracts, homogenates, and tissue slices has provided much detailed information concerning the occurrence and components of complex enzyme systems, such as the glycolytic and tricarboxylic acid systems. Various inhibitors were used in this study in order to assess the importance of these enzyme systems for the metabolism of islet tissue.

TABLE IV

Effect of glutamate on the respiration of toadfish islet and liver slices

Medium	Tonicity of medium (equiv. NaCl conc. *M*/liter)	Islet			Liver		
		No. of deter.	Ave. mμl. O_2/μg. prot./hr.	σ	No. of deter.	Ave. mμl. O_2/μg. prot./hr.	σ
0.054 *M* PO_4	0.0755	14	1.6	0.66	9	2.2	0.40
0.054 *M* PO_4 + 0.0645 *M* NaCl	0.140	11	1.6	0.64	9	2.3	0.23
0.054 *M* PO_4 + 0.043 *M* glutamate	0.140	13	2.3	0.78	9	3.0	0.30

Table VII shows the per cent inhibition produced by the various inhibitors. Fluoride added to a phosphate medium was found to inhibit respiration of islet slices by 35% ($p = 0.0063$), iodoacetate inhibited by 53% ($p = <0.001$), malonate by 43% ($p = <0.001$), and azide by 50% ($p = 0.001$).

DISCUSSION

Although the addition of glucose did not stimulate islet tissue respiration, this does not mean that glucose can not be utilized by islet tissue. There are a number of reasons why an added substrate might not stimulate respiration, such as its failure to readily enter the cell or the presence of sufficient endogenous substrate to saturate the enzyme system concerned. This is emphasized by our finding that

TABLE V

Effect of succinate on the respiration of toadfish islet and liver slices

Medium	Islet				Liver			
	Tonicity of medium (equiv. NaCl conc. *M*/liter)	No. of deter.	Ave. mμl. O_2/μg. prot./hr.	σ	Tonicity of medium (equiv. NaCl conc. *M*/liter)	No. of deter.	Ave. mμl. O_2/μg. prot./hr.	σ
0.054 *M* PO_4	0.0755	14	1.1	0.61	0.0755	9	2.9	0.37
0.054 *M* PO_4 + 0.0645 *M* NaCl	0.140	14	1.5	0.64	0.140	9	2.7	0.37
0.054 *M* PO_4 + 0.0645 *M* succinate	0.172	13	2.2	0.77	0.140*	9	4.9	0.89

* 0.043 *M* succinate used in this case.

the addition of glucose did not stimulate liver respiration, despite the known occurrence of the glycolytic system in the liver of most species. Our studies with fluoride and iodoacetate provide suggestive evidence that at least two enzymes of the glycolytic scheme (enolase and phosphoglyceraldehyde dehydrogenase) are also present in islet tissue. Neither of the inhibitors can be considered specific, but phosphoglyceraldehyde dehydrogenase is extremely sensitive to iodoacetate (Meyerhoff and Kiessling, 1933; Adler, Euler and Gunther, 1938) and at least one of the actions of fluoride is to inhibit enolase (Warburg and Christian, 1941).

Of the enzymes involved in the tricarboxylic acid cycle, the presence of α-ketoglutarate oxidase and succinoxidase are indicated by the stimulation of oxygen uptake observed following the addition of their respective substrates. The presence of succinoxidase is further indicated by the observed inhibition by malonate (43%). This inhibition can be compared with the 50–60% inhibition of rat liver homogenates reported by Holtkamp and Hill (1951) and Pardee and Potter (1949) as well as the 70% inhibition of rat brain homogenates (using 0.02 *M* malonate) reported by Pardee and Potter (1949). Finally, the participation of succinoxidase in islet tissue respiration is also consistent with the previous demonstration of the presence of succinate-cytochrome *c* reductase in this tissue (Lazarow and Cooperstein, 1951).

Neither pyruvate nor isocitrate stimulated islet respiration but this could be due to failure of these substrates to penetrate to their site of utilization. The

TABLE VI

Effect of isocitrate on the respiration of toadfish islet slices

Medium	Tonicity of medium (equiv. NaCl conc. *M*/liter)	No. of deter.	Ave. mμl. O_2/μg. prot./hr.	σ
0.054 *M* PO_4	0.0755	12	2.2	0.58
0.054 *M* PO_4 + 0.645 *M* NaCl	0.140	12	2.2	0.57
0.054 *M* PO_4 + 0.032 *M* isocitrate	0.140	12	2.3	0.45

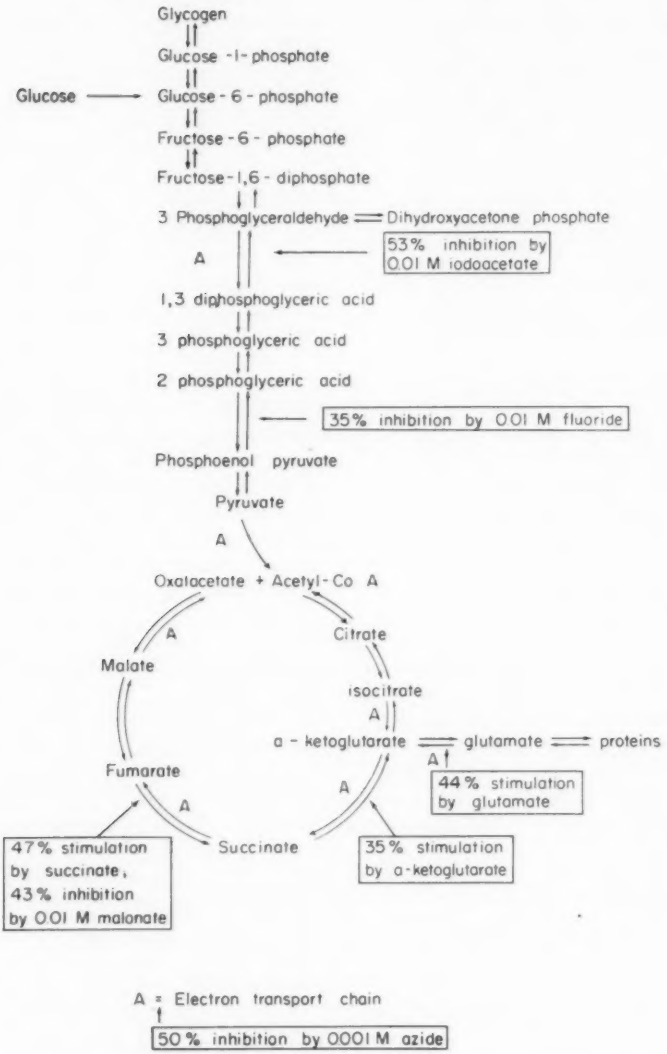

FIGURE 1. A simplified scheme of carbohydrate metabolism, which indicates the evidence obtained for its operation in islet tissue metabolism.

TABLE VII

Effect of inhibitors on the respiration of toadfish islet tissue. (Each inhibitor was studied in a separate series of experiments and compared with controls run at the same time)

Medium	No. of deter.	Ave. mμl. O_2/ μg. prot./hr.	σ	% inhibition
0.054 *M* PO_4	13	2.0	0.85	
0.054 *M* PO_4 + 0.01 *M* fluoride	14	1.3	0.37	35
0.054 *M* PO_4	16	1.9	0.74	
0.054 *M* PO_4 + 0.01 *M* iodoacetate	13	0.9	0.45	53
0.054 *M* PO_4	12	2.1	0.43	
0.054 *M* PO_4 + 0.01 *M* malonate	12	1.2	0.37	43
0.054 *M* PO_4	8	2.0	0.79	
0.054 *M* PO_4 + 0.001 *M* azide	8	1.0	0.24	50

enzymes of the tricarboxylic acid cycle are localized within the mitochondria, and it has been reported (Schneider, Striebich and Hogeboom, 1956) that the mitochondrial membrane may not be permeable to citrate.

The ability of islet to utilize glutamate is of considerable interest since this compound is a key link between the tricarboxylic acid cycle and protein synthesis.

The existence of cytochrome oxidase in islet tissue has been reported previously (Lazarow and Cooperstein, 1951) and its participation in the electron transport system is indicated by the inhibition of respiration produced by azide,[4] a known inhibitor of cytochrome oxidase (Keilin, 1936).

Figure 1 depicts a simplified scheme of carbohydrate metabolism, and indicates the evidence obtained for its operation in islet tissue metabolism.

SUMMARY AND CONCLUSIONS

The effects of substrates and inhibitors on the metabolic activity of toadfish islet slices were measured in the Cartesian diver microrespirometer under varying experimental conditions. Of the substrates added, α-ketoglutarate, glutamate, and succinate increased islet respiration while glucose, pyruvate, and isocitrate were ineffective. Iodoacetate, fluoride, malonate, and azide inhibited islet respiration.

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[4] Studies with cyanide have not been carried out to date because these are very difficult to perform in the Cartesian diver.

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A STUDY OF REPRODUCTION IN THE INTERTIDAL BARNACLE, MITELLA POLYMERUS, IN MONTEREY BAY, CALIFORNIA[1, 2]

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The goose barnacle, *Mitella polymerus* (Sowerby, 1833), sometimes referred to as *Pollicipes polymerus* or the leaf barnacle, is distributed along the exposed rocky coast of Western North America from British Columbia to the middle of Baja California (Cornwall, 1925). It is generally abundant here in the upper two-thirds of the intertidal belt (Ricketts and Calvin, 1952), though it is occasionally found below this level where there is considerable surging wave action. Along the central California coast, clusters of *Mitella* patch the exposed rocky regions, the barnacles usually attaching themselves to rock, to *Mytilus californianus*, or to other individuals of *Mitella*. Individuals are seldom isolated and one sees among the mussel beds or on the rocks rosette-shaped clusters in which large barnacles are at the center and smaller barnacles grade toward the periphery. In other aggregations, individuals of nearly the same size are packed closely together, frequently with such uniformity in size and orientation that their valves form a geometric pattern, and their closely packed bodies make a strong but resilient mat against the pounding surf. Often where the animals are attached to rock beneath the mussel beds, their stalks extend up eight inches or more to the surface. Occasionally, solitary animals occur on tables of rock exposed to strong wave action; in these the stalks remain short and stubby while the shells and bodies grow.

While *Mitella polymerus* is abundant, conspicuous, and well-known taxonomically, almost nothing is known of its reproductive biology. Nussbaum (1890) has described the anatomy of *M. polymerus* in some detail. The only published study of reproduction and development in this genus is that of Batham (1944–45), on the New Zealand species, *Mitella spinosus*. Since Batham's study of the reproductive cycle was carried out at latitude 44°52′ South, a comparison of her results with the situation occurring in *M. polymerus* at Monterey Bay (36°40′ North) seemed particularly interesting. The following study of reproduction in *M. polymerus* includes: the anatomy of the reproductive system, the relationship between size and sexual reproduction, the seasonal reproductive cycle, the rate of egg and embryo development, an estimate of fecundity, and evidence concerning self-fertilization.

[1] This investigation was carried out as partial fulfillment for the degree, Master of Arts.

[2] The author wishes to express her gratitude to Dr. Donald P. Abbott, under whose guidance this work was carried out; and to acknowledge with appreciation his generous help with arrangement and discussion of the data.

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The Reproductive System

The gross reproductive anatomy of *Mitella polymerus* is shown in Figure 1. Ovarian tissue is found in the upper portion of the peduncle. From the ovaries, a pair of oviducts lead up into the body proper, emptying into the glandular oviducal atria in the bases of the first thoracic cirri. Eggs pass down the oviducts to the

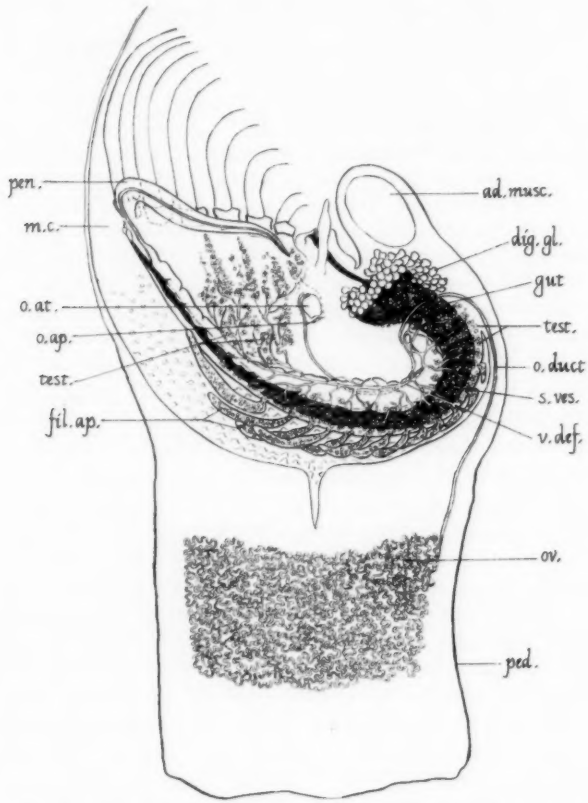

FIGURE 1. Gross anatomy of the reproductive system of *M. polymerus*, exposed by dissection from the left side. ad. musc. = adductor muscle; dig. gl. = digestive gland; fil. ap. = filamentary appendages; gut = gut; m. c. = mantle cavity; o. ap. = oviducal aperture; o. at. = oviducal atrium; o. duct = oviduct; ov. = ovaries; ped. = peduncle; pen. = penis; test. = testes; s. ves. = seminal vesicle; v. def. = vas deferens.

atria, where they receive a protective coating which glues them together into masses. The egg masses are then extruded through the oviducal apertures and come to lie in the mantle cavity on either side, where they are pressed flat to form the two ovigerous lamellae.

The numerous small testes are found on either side of the gut, extending ventrally into the coxopodites of the first four pairs of thoracic appendages and dorsally into the numerous paired filamentary appendages. Fine efferent ducts connect the testes to the paired vasa deferentia, which lead to the paired storage organs or seminal vesicles. These in turn join posteriorly at the base of the penis, and form a single duct extending to its tip.

Copulation was not observed, but sperm are deposited in the mantle cavity. The embryos are brooded in the ovigerous lamellae in the mantle cavity until they are hatched out as nauplius larvae.

The gross reproductive anatomy of *M. polymerus* is similar to that of *M. spinosus;* but *M. spinosus* has no filamentary appendages and the testis is described as (p. 370) "a median structure lying closely in the U-bend of the gut," with a pair of ducts leading from either side of it which expand to form the seminal vesicles (Batham, 1944–45).

Materials and Methods for the Study of Reproduction

Nearly all living material used in the present study was collected in or very near Monterey Bay, California, and studies were carried out at the Hopkins Marine Station of Stanford University, Pacific Grove.

For the study of the reproductive cycle, a population of *Mitella polymerus* was sampled at approximately monthly intervals for a period of fifteen months. All individuals were collected within an area approximately fifteen feet square on granite rocks and adjacent beds of *Mytilus californianus* on the northern shores of Mussel Point, Pacific Grove, California, at an intertidal level corresponding to the upper middle horizon of Zone Three of Ricketts and Calvin (1952). Care was taken to insure that all barnacles large enough to be reproductively mature were collected in close proximity to other individuals of a reproductive size, and to avoid taking isolated individuals which had lacked the opportunity for cross-fertilization.

Animals were anaesthetized for four hours in a solution of magnesium chloride isotonic with sea water. This was sufficient to relax the cirri and peduncle. The animals were then preserved in 10% formalin buffered with borax.

Individuals of all sizes were collected and examined. For most of the reproductive data, the largest common animals available were used. All of these were of a reproductive size, and for purposes of consistency, large animals which ranged in breadth (distance from rostrum to carina) from 27.5 mm. to 32.5 mm. were used (occasionally larger animals are found).

When maturing, the ovarian tissue in the peduncle (Fig. 1) undergoes marked and visible changes. Tiny eggs appear and grow, accumulating yolk until they are passed up through the oviducts and are extruded into the mantle cavity. Preliminary observations showed that throughout the ovarian tissue, eggs are generally of about the same size and stage of development at any one time; an exception to this is provided where a new batch of tiny eggs appears in the ovary before the previous batch of very much larger eggs is extruded. With a compound microscope and calibrated ocular micrometer, the greatest diameters of five to ten eggs were measured (in each animal), and an average ovarian egg size was recorded for the individual. Where two batches of eggs were present at once in

an ovary (*i.e.*, large and small), this situation was quite evident, and the two were treated separately. The average egg sizes were then grouped into three useful classes for purposes of comparison: small eggs (diameter 0.016 mm. to 0.065 mm.), medium eggs (diameter 0.066 mm. to diameter 0.099 mm.), and large eggs (diameter 0.100 mm. to diameter 0.127 mm.). The few individuals in which no eggs could be seen were treated separately.

As the male gonads of *Mitella* mature, they also show easily visible changes: testes in the filamentary appendages and throughout the body lose their translucency and become opaque white with sperm. Sperm then travel through efferent tubules and vasa deferentia to the paired seminal vesicles, where they are stored. As more and more sperm accumulate in the seminal vesicles, these, too, change from translucent to opaque white and increase in diameter. Diameter of the seminal vesicles affords a fairly good index for the maturation of the male reproductive organs. Width was measured with a pair of dividers at a point just back of the anterior sharp bend in the seminal vesicle (Fig. 1). Conditions of the male organs were finally grouped into three categories: (1) seminal vesicles translucent and apparently empty of sperm; (2) seminal vesicles ranging in width from one to two mm.; and (3) seminal vesicles more than two mm. in width (ranging up to a maximum observed width of 3.8 mm.).

Fertilized eggs and developmental stages present in ovigerous lamellae were also studied. Embryos were examined in the ovigerous lamellae from animals taken in the monthly samples. In a pair of ovigerous lamellae taken from any one parent, embryos are all at about the same stage of development. As the fertilized egg develops, it increases slightly in size, but not enough for size to yield a good criterion for stage of development. Major morphological changes can be studied fairly readily, and it proved practicable for the purpose of the present problem to divide embryonic development into three stages: (1) early stages, with neither limb buds nor nauplius eye; (2) middle stages, with limb buds developing but no nauplius eye; and (3) late stages, with well-formed limbs and median eye present. Studies were also made of lamellae removed from the parent and raised *in vitro*. Ovigerous lamellae were isolated from their parent barnacles, placed in clear glass dishes of filtered sea water, and kept at a constant 13° C. in a water bath or a refrigerated room. Sea water was changed approximately daily, at which times the embryos were examined in small sections of the ovigerous lamellae plucked off with glass needles. Many embryos raised *in vitro* were observed through development to hatching, and many larvae were raised beyond this through several naupliar molts.

Size and Sexual Reproduction

Figure 2 shows the occurrence of ovigerous lamellae in animals of different sizes at selected times during the breeding season (May through December). Animals with a breadth of more than 27.5 mm. were always found to be sexually mature during the reproductive season; all individuals examined bore ovigerous lamellae, or enlarging ova in the ovaries, or both. In the individuals below 27.5 mm. breadth, the percentage of animals with ovigerous lamellae can be seen to decrease more or less directly with decreasing size. For individual months, the tendency is not always clear, and this may be due to sampling deficiencies (*e.g.*,

class III in July and September). However, the summary graph, which represents values for the total number of animals of each size class, points out the trend well, and the differences shown here are statistically significant. The variation in reproductive activity with size may be explained in either of two ways: possibly a lower percentage of the smaller animals are sexually mature; or (less likely) the smaller class animals produce ovigerous lamellae less frequently than the larger animals. No ovigerous lamellae were ever found in any barnacle with a breadth of less than 17.2 mm.

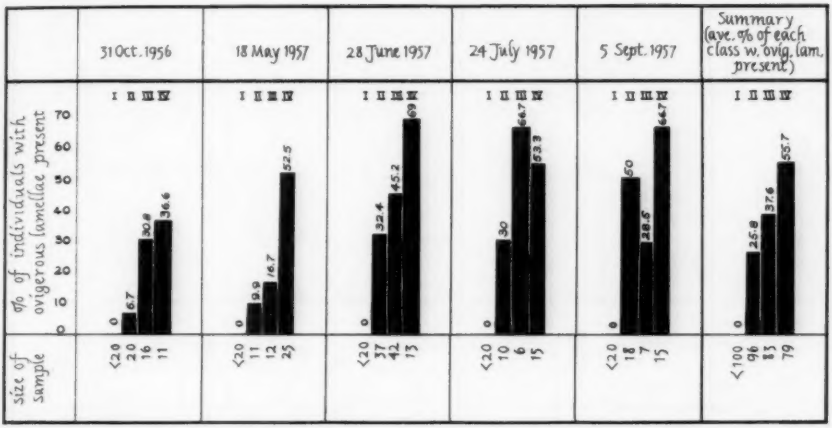

FIGURE 2. Variation in reproductive activity with size of animals. Vertical bars indicate percentage of individuals of each size class which contained ovigerous lamellae. Size classes are designated as follows: I, breadth less than 17.2 mm.; II, breadth 17.2 to 22.5 mm.; III, breadth 22.6 to 27.5 mm.; and IV, breadth 27.5 to 32.5 mm.

THE REPRODUCTIVE CYCLE IN THE MITELLA POLYMERUS POPULATION

Each month, 10 to 25 large animals were collected, measured, and examined for reproductive condition. The number of individuals carrying ovigerous lamellae was noted; conditions of the female and male gonads, and stage of development of the embryos (where present) were studied.

The distribution of egg sizes and larval stages is shown in Table I and Figure 3. Table I shows for each sample the number and per cent of *individuals* which contained given egg size classes and which brooded particular embryonic stages. However, in Figure 3, each separate mass of eggs or embryos is treated as a separate unit. For example, in cases where a given individual contained simultaneously small eggs, large eggs, and late embryos, these appear in Figure 3 in three different horizons in the same date column.

It is apparent in Figure 3 that between November, 1956, and January, 1957, breeding waned and was discontinued until the spring of 1957. Then, during March, eggs began to enlarge, but did not exceed the upper limits of the class of small eggs. By April, the first medium and large ovarian eggs were present, and

the first fertilized egg mass appeared. Egg production continued through the year to January, 1958, when the number of ovigerous lamellae present in the population dropped significantly. The season of reproductive activity for the population covers three-quarters of the year, and it is of interest that all stages of egg and embryo development were found throughout the season. On the average, during the height of the breeding season, between 50% and 60% of the population of large animals are carrying ovigerous lamellae at any one time.

Data for the male gonads are plotted in Table I and in Figure 4. Sperm is present in at least some members of the population throughout the year. While there is seasonal variation in the condition of the male gonads, this is less well defined than is that of the female gonads. In the fall of 1956 and through January,

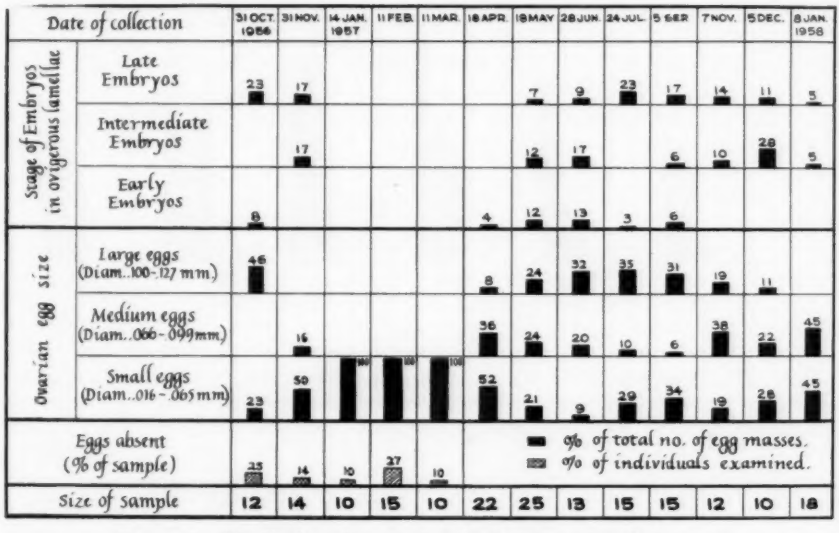

FIGURE 3. Distribution of egg and embryo masses of various stages of development in large *M. polymerus*.

1957, some seminal vesicles were very thick with sperm, while others appeared spent. In February and March, 1957, most of the vesicles had a meager amount of sperm, presumably building up, and some were empty. In April, most vesicles were quite full with sperm, others had a meager amount, and none were empty, and from this point on through the summer and fall of 1957, the mean vesicle width was high (well over two mm.). Empty vesicles were not observed again until January, 1958. Thus during the winter months, the quantity of sperm present in the seminal vesicles of most of the population was considerably less than the amount present during the rest of the year.

There is a suggestion of waves of reproductive activity during the breeding season, in the data presented in Figure 3 and Table I. This appears most clearly in Table I in the column showing the percentage of animals which are carrying

TABLE I

Seasonal variation in egg size, seminal vesicle width, and stage of development of brooded embryos in M. polymerus

Dates	1956 Oct. 31	Nov. 30	1957 Jan. 19	Feb. 11	Mar. 11	Apr. 18	May 18	Jun. 28	Jul. 24	Sept. 5	Nov. 7	Dec. 5	1958 Jan. 8
No. of animals examined:	12	14	10	15	10	22	25	13	15	15	12	10	18
No. and per cent of animals with:													
Eggs absent													
No.	3	2	1	4	1	—	—	—	—	—	—	—	—
%	25	14	10	27	10	—	—	—	—	—	—	—	—
Small eggs													
No.	3	9	9	11	9	11	5	1	1	2	—	4	9
%	25	65	90	73	90	50	20	8	7	13	—	40	50
Medium eggs													
No.	—	3	—	—	—	9	10	6	3	2	8	4	9
%	—	21	—	—	—	41	40	46	20	13	67	40	50
Large eggs													
No.	6	—	—	—	—	—	6	5	3	1	—	1	—
%	50	—	—	—	—	—	24	38	20	7	—	10	—
Both small and Large eggs													
No.	—	—	—	—	—	2	4	1	8	10	4	1	—
%	—	—	—	—	—	9	16	8	53	67	33	10	—
No. and per cent of animals with ovigerous lamellae:													
No.	4	6	—	—	—	1	13	9	8	10	5	7	2
%	33	43	—	—	—	5	52	69	53	67	42	70	11
No. and per cent of animals with ovigerous lamellae bearing:													
Early embryos													
No.	1	—	—	—	—	1	5	3	1	2	—	—	—
%	8	—	—	—	—	5	20	23	7	13.5	—	—	—
Middle embryos													
No.	—	3	—	—	—	—	5	4	—	2	2	5	1
%	—	21.5	—	—	—	—	20	31	—	13.5	17	50	5.5
Late embryos													
No.	3	3	—	—	—	—	3	2	7	6	3	2	1
%	25	21.5	—	—	—	—	12	15	46	40	25	20	5.5
Conditions of seminal vesicles present in the population													
No. of animals examined:	12	13	18	15	10	21	23	13	14	14	12	10	18
No. of animals with seminal vesicles in following conditions:													
Apparently empty	1	1	1	4	3	—	—	—	—	—	—	—	2
Width 1–2 mm.	5	6	5	9	7	4	1	2	2	5	4	9	7
Width 2–3.8 mm.	6	6	12	2	—	17	22	11	12	9	8	1	9

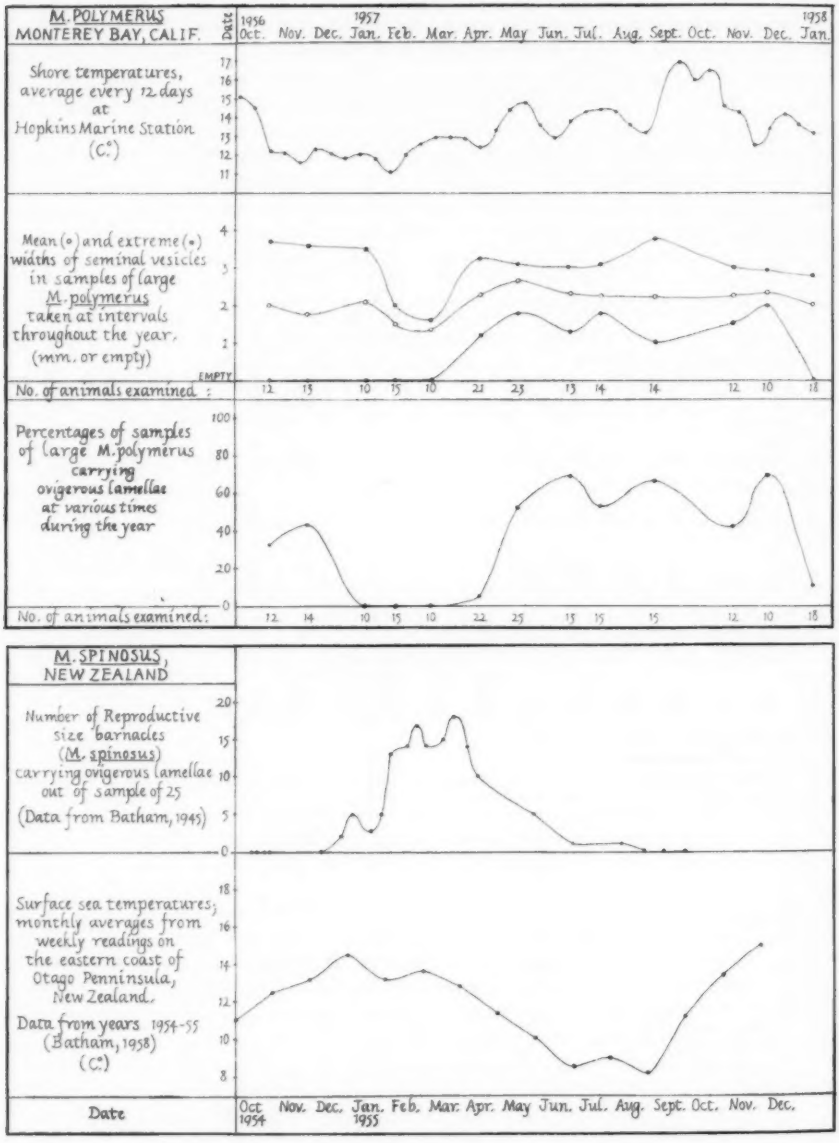
M. POLYMERUS
MONTEREY BAY, CALIF.
Date
1956 1957 1958
Oct. Nov. Dec. Jan. Feb. Mar. Apr. May Jun. Jul. Aug. Sept. Oct. Nov. Dec. Jan.
Shore temperatures, average every 12 days at Hopkins Marine Station (C°)
17
16
15
14
13
12
11
Mean (o) and extreme (•) widths of seminal vesicles in samples of large M. polymerus taken at intervals throughout the year. (mm. or empty)
4
3
2
1
EMPTY
No. of animals examined :
12 13 10 15 10 23 25 13 14 14 12 10 18
Percentages of samples of large M. polymerus carrying ovigerous lamellae at various times during the year
100
80
60
40
20
0
No. of animals examined:
12 14 10 15 10 22 25 13 15 15 12 10 18
M. SPINOSUS, NEW ZEALAND
Number of Reproductive size barnacles (M. spinosus) carrying ovigerous lamellae out of sample of 25
(Data from Batham, 1945)
20
15
10
5
0
Surface sea temperatures; monthly averages from weekly readings on the eastern coast of Otago Peninsula, New Zealand.
Data from years 1954-55 (Batham, 1958)
(C°)
18
16
14
12
10
8
Date
Oct. 1954 Nov. Dec. Jan. 1955 Feb. Mar. Apr. May Jun. Jul. Aug. Sept. Oct. Nov. Dec.

FIGURE 4. Temperature and reproductive activity in *M. polymerus* in Monterey Bay and in *M. spinosus* in New Zealand.

ovigerous lamellae at different times during the year. The highest values, *i.e.*, those for June, September, and December (69%, 67%, and 70%, respectively), alternate with the lower values shown for May, July, and November (53%, 53%, and 42%, respectively), and therefore suggest that the population as a whole may be reproducing in waves, more or less simultaneously. As will be indicated later, this phenomenon is very likely related to the successive waves of reproductive activity shown by *individual* barnacles during the breeding season. However, not all individuals start to reproduce at precisely the same time, and waves of activity do not proceed in all individuals at exactly the same rate. The differences between alternate highs and lows and their deviations from the mean for the population are not statistically significant; and a larger sample might be expected to yield a smoother plateau for reproductive activity of the population.

The Reproductive Cycle in Individuals

The reproductive cycle in the population of *M. polymerus* represents a summation of the reproductive processes in individual barnacles. The animals are hermaphroditic and ovoviviparous, brooding their young for a period of time in the mantle cavity. It is accordingly of interest to examine the relative degrees of development of the two gonads in individual animals, and to relate the ovarian egg size with the stage of development of brooded embryos present simultaneously in individual barnacles.

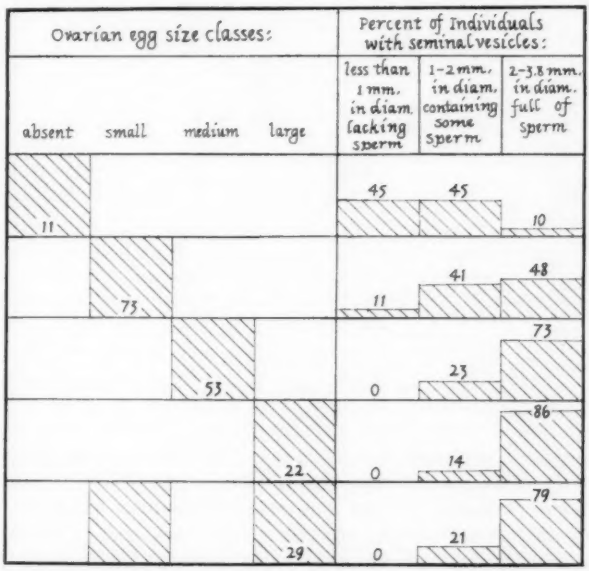

Figure 5. Egg sizes and seminal vesicle widths found simultaneously in large individuals. Left-hand side: numbers within the blocks represent numbers of individuals examined; right-hand side: numbers above blocks represent percentages of individuals with seminal vesicles of given sizes.

For studies of the two gonads, the large animals taken at intervals throughout the study period were examined. Animals were grouped into four categories according to egg size: animals with small eggs, those with medium eggs, those with large eggs, and those with both small and large eggs. The seminal vesicle width of each animal of a given egg category was noted. The data for all animals examined are shown in Figure 5.

In Figure 5, the bars on the right hand side represent the frequency of occurrence of the various conditions of the seminal vesicle which may be found in indi-

TABLE II

Correlation of size of ovarian egg with developmental stage of brooded embryos present in the same individual. Numbers of individuals showing each combination of conditions are indicated by numbers within the squares. Heavy vertical lines enclose the "peak" months of the breeding season.

Egg size	Embryos	31 Oct.	30 Nov.	14 Jan.	11 Feb.	11 Mar.	18 Apr.	18 May	28 June	24 July	5 Sept.	7 Nov.	5 Dec.	8 Jan.
Small & Large	Absent						2	4	1	4	6	3	1	
	Early													
	Middle													
	Late									5	4	1		
Small	Absent	2	5	9	11	9	11			1				8
	Early							4	1		1			
	Middle		2					1			1		3	
	Late	1	2										1	1
Medium	Absent		1				9	3				4	1	8
	Early							1	1	1				
	Middle		1					4	4		1	2	2	1
	Late		1					2	1	1	1	2	1	
Large	Absent	4						5	4	2			1	
	Early													
	Middle													
	Late	2						1	1	1	1			
Eggs Absent	Absent	3	2	1	3	1								
	Early													
	Middle													
	Late													
		12	14	10	14	10	22	25	13	15	15	12	10	18

viduals with eggs of any given size category. We see from the graph that sperm is absent only in animals where eggs are small or absent. Under all other ovarian conditions, sperm is always present, and in the majority of barnacles with medium or large eggs, the seminal vesicles are full.

Table II contains data illustrating the conditions which may occur simultaneously in the ovary and in the brooded ovigerous lamellae, in the same individual at different times of the year. From these data, the information on large animals collected during the breeding season (October to November, 1956; and May, 1957

to January, 1958) and containing both ovarian eggs and ovigerous lamellae, has been selected and is shown graphically in Figure 6.

It is evident from Figure 6 that there is a general relationship between ovarian egg size and brooded embryo stage in the same individual, particularly during the height of the breeding season. In general, where eggs are small in the ovaries, embryos are in an early stage of development in the mantle cavity; where eggs are medium-sized in the ovaries, embryos are in the middle stage of development in the mantle cavity; and where eggs in the ovaries are large (nearly ready to be extruded), embryos in the mantle cavity are advanced (nearly ready to be liberated). The parallel diagonal lines drawn through successive peak conditions show clearly the synchronized pattern of development of ovaries and embryos during the peak months of the reproductive season. Certainly such a condition, in which eggs and embryos show more or less parallel development, appears to

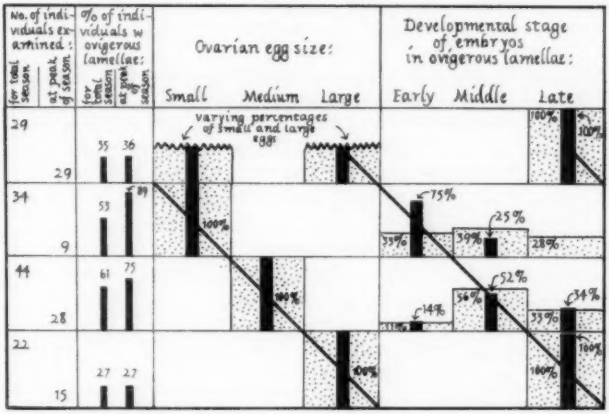

FIGURE 6. Developmental stages, within individuals, of ovarian eggs and brooded embryos, where both are present simultaneously. Numbers pointing to black bars show percentages of given stages present during the "peak" months of the reproductive season; numbers within the larger bars show percentages of given stages present during the total reproductive season.

represent the most efficient timing pattern for an ovoviviparous organism which produces successive broods of young in a single breeding season. However, it is clear from the graph and the table that precise coordination between rates of egg enlargement and larval development does not exist for all animals examined during the reproductive season. For example, some animals with small eggs in the ovaries were found brooding middle or late stage embryos. In such cases, it appears that the interval between successive broods of larvae is greater than in cases where eggs and embryos develop in phase, and that ovarian egg development is retarded with respect to embryonic development. Table II shows clearly that these animals with relatively retarded ovarian development tend to be localized in time, predominating during the later months and not the peak months of the reproductive season. Thus, the five cases in which late embryos accompany small

ovarian eggs occurred in October and November of 1956, December of 1957, and January of 1958, suggesting that at this time development of the ovaries had slowed down or stopped for the season, and that the final batch of nauplii would soon have been shed. Also, the combination of small eggs with middle embryonic stages occurred, with one exception, either at the beginning (May, 1957) or near the end (November, 1956 and December, 1957) of the breeding season. Animals with medium eggs and *no* ovigerous lamellae occurred in April and May, 1957 (when they were presumably giving rise to their first batch of eggs of the season) and in November, 1956 and 1957 (when they may represent individuals in which the ovarian growth has slowed down toward the close of the season). In contrast, a few animals contained medium-sized eggs along with early embryos in the ovigerous lamellae, suggesting a relative acceleration of egg development. However, in all three cases, the eggs measured fell close to the lower size limits of the medium egg size class, and the apparent acceleration is exaggerated by the positioning of size-class limits in the grouping of data.

TABLE III

Apparent conditions of ovaries (ovarian eggs) with relation to embryos (found simultaneously in an individual)

Dates	Number of times ovaries appeared in following conditions:		
	Accelerated	Synchronized	Retarded
31 Oct. 1956	—	2	1
30 Nov. 1956	—	1	5
14 Jan. 1957 11 Feb. 1957 11 Mar. 1957 18 Apr. 1957	no reproductive activity		
18 May 1957	1	9	3
28 Jun. 1957	1	6	1
24 Jul. 1957	1	6	1
5 Sept. 1957	—	7	2
7 Nov. 1957	—	3	2
5 Dec. 1957	—	2	5
8 Jan. 1958	—	1	1

Table III summarizes the condition of the ovaries with relation to embryonic stages. We see that during the early and middle months of the reproductive season, most of the animals examined appeared to show synchrony in rates of ovarian and embryonic development. Toward the end of the reproductive season, a relative lag in ovarian egg development became noticeable. By combining data for the months of May through September (the main reproductive period) and for the period of October through December (the end of the breeding season), we can show that the prevalence of animals with synchronized ovaries during the former months, and of animals with retarded ovaries during the latter months, is indeed statistically significant.

While animals with apparently well synchronized brood development are probably producing batches of eggs at a relatively efficient rate, there is evidence that even in such animals some time does elapse between broods. We might expect, in a perfectly synchronized animal, that immediately following the liberation of a batch of larvae, a second batch of eggs would be extruded, and that such an animal would be carrying ovigerous lamellae virtually all of the time. However, at no time during the reproductive period were all reproductively mature members of the population carrying ovigerous lamellae; even during the height of the breeding season, only 50% to 60% of the population were brooding embryos. The occurrence of a very few slow developers during this time could hardly account for such a discrepancy; and it must be assumed that even during the height of reproduction, some time does elapse between the hatching of a batch of larvae and the extrusion of the next batch of eggs from the same individual.

Number of Broods and Larvae Liberated by Mitella polymerus, and its Relative Fecundity

No direct observations are available on the number of broods produced by an adult barnacle per year. However, by combining all the lines of evidence at hand, we can arrive at a hypothetical figure for the number of batches of larvae liberated by a large individual during one reproductive season. Three lines of evidence are considered here: the duration of the reproductive season, the rate of development of embryos in the ovigerous lamellae, and the developmental stages of eggs and embryos present at any one time in an individual during the reproductive season.

We have already seen that the reproductive period for most barnacles extends through about eight months or about 240 days (Fig. 3). The approximate duration of the lamellar brood period was determined in the laboratory. Several batches of larvae were raised *in vitro* at 13° C., starting with what appeared to be the two-cell stage, and continuing to hatching. Periodic examination of these ovigerous lamellae showed that embryos remain in the "early" stage for the first nine days, are in the "middle" stage from the tenth through the fifteenth day, and remain in the "late" stage from the sixteenth day to hatching, which occurs on the twenty-ninth to the thirty-first day. Development from fertilization to hatching of the nauplius thus averages about thirty days. This rate is substantiated for field conditions in Figure 3, where we find the first large eggs being extruded in April and the first late embryo just one month later. It is interesting to note that the brood period for "normal" *M. spinosus*, raised under conditions where temperature was not controlled but averaged 14° C. to 15° C., was thirty to thirty-two days (Batham, 1946).

The developmental stages present simultaneously in individuals have been shown in Figure 6 and Table II. Here, the simultaneous presence in single animals of three stages (small eggs, large eggs, and late embryos, especially in animals taken in July and September) strongly suggests that an animal may give rise to *at least* three batches of embryos during one reproductive season. Tables II and III show that during the first six months of the reproductive season, egg and embryo stages tended to be more or less synchronized in individuals; and during the last two months of the breeding season, the majority of animals carried broods which tended to be out of phase by two stages. In a perfectly synchronized animal, in which

ovarian eggs and lamellar embryos develop in phase, we might assume that the interval between the average small egg and the average early embryo is equal to the lamellar brood period, or thirty days. In animals one step out of phase, that is, in animals in which *medium* embryos accompany *small* eggs, or *late* embryos accompany *medium* eggs, we might assume that the interval between broods is thirty-eight days (or thirty days plus the difference between the average age of an early embryo and the average age of a middle stage embryo—eight days). In animals two steps out of phase, in which *late* embryos accompany *small* eggs, or (specifically in November, 1957) *no* embryos accompany *medium* eggs, we might assume that the interval between successive broods is at least 48.5 days (or thirty days plus the difference between the average age of an early embryo and the average age of a late embryo—18.5 days).

Through the breeding season (eight months), these various intervals were found to be more or less localized in time. That is, for approximately the first six months, most of the population seemed to be producing broods either in phase or one stage out of phase. Hence, at the assumed rate of one brood for every thirty to thirty-eight days, a minimum of 4.7 broods and a maximum of six broods could have been extruded by an individual during this time. For the last two reproductive months, a majority of the population carried broods two steps out of phase. At the assumed rate, then, of one brood for every 48.5 days, one to 1.2 broods may have been extruded by an individual during these two months. During the TOTAL reproductive period, then, it appears that a single large barnacle *could* have liberated from five to 7.2 broods of larvae.

If an average animal gives rise to six broods in a season, each brood developing in the mantle cavity for thirty days, we would expect such an animal to be carrying embryos for approximately 180 days out of the total 240 days, or approximately 75% of the total time. Since no single individual was followed during the breeding season, no data are available to provide a direct means of checking this figure. However, 59% of the large individuals collected during the eight-month reproductive season bore ovigerous lamellae. This suggests that perhaps large individuals contain ovigerous lamellae for only about 59% of the total reproductive period, or 142 out of 240 days. On this basis, then, the average animal probably produced only four or five broods during the season.

There seem to be two good reasons for the discrepancy between the two estimates for average number of broods per season. The first, already mentioned, is that even in well-synchronized animals, some time did elapse between successive lamellar broods. There was no direct measurement of the duration of this minimum interval, and thirty days was used as the minimum time for the enlargement and extrusion of eggs; but actually, the period during which eggs remain in the ovary is probably a few days longer. A suggestion providing some independent support for this is seen in the data in Figure 3, where on March 11, 1957, all ovarian eggs were in the small size class (though some approached the upper limits of this class), and on April 18, 1957, 36 days later, one individual was just extruding eggs from the oviduct. The other possible reason for the discrepancy between the two estimates of number of broods produced per year is shown by the fact that many stages of development were found in the population at any one time, and that the whole population did not produce broods synchronously. It is probable

that the average animal did not reproduce at the given rates for a total of 240 days; that animals may have either started reproducing later than the earliest assumed limit or stopped reproducing earlier than the latest assumed limit. Such animals may have produced only five or four, or possibly only three broods during a season. In contrast, the results of Batham (1944–45) show that *M. spinosus* in New Zealand probably liberates only two per season.

Counts have been made of the number of larvae contained in the ovigerous lamellae of both species of *Mitella*. It was found that a large *M. polymerus* may liberate from 104,000 to 240,000 larvae from a single brood contained in one pair of ovigerous lamellae; the slightly smaller *M. spinosus* liberates approximately 3000 larvae per brood (Batham, 1944–45). It appears that a single adult *M. polymerus* may produce roughly 52 to 280 times as many larvae per year as a single adult *M. spinosus*.

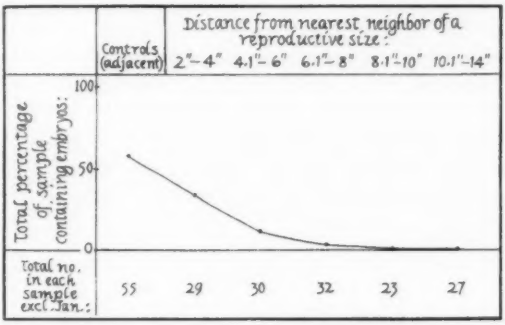

FIGURE 7. Summary graph showing percentages of large *M. polymerus* containing ovigerous lamellae when separated from other large specimens by given distances.

SELF-FERTILIZATION

For a study of self-fertilization, a series of large and relatively isolated barnacles, situated at various measured distances from their nearest neighbors of reproductive size, were collected and inspected for the presence of ovigerous lamellae. These samples were collected at different times throughout the reproductive season. The data, grouped for the months of collection, and for the distances by which the sexually mature barnacles were separated, are presented in Table IV and Figure 7. It can be seen that over 50% of the control animals for each month (except January, 1960) carried ovigerous lamellae; animals separated by two to four inches appeared to carry ovigerous lamellae less frequently, but the differences are not statistically significant. There is a statistically significant drop in the presence of embryos in animals separated from their nearest neighbor of a reproductive size by over four inches, and an absence of embryos in animals separated by more than eight inches. Thus it appears that eight inches was the maximum distance over which copulation of large animals collected could take place, and that animals separated by greater distances failed to receive sperm. Despite the

TABLE IV

Numbers of barnacles of reproductive size isolated from other such barnacles by given distances, showing presence (+) and absence (−) of ovigerous lamellae

Date	Distance from nearest neighbor of reproductive size:											
	Controls (adjacent)		2″–4″		4″–6″		6″–8″		8″–10″		10″–14″	
	+	−	+	−	+	−	+	−	+	−	+	−
1958	12	8	3	2	1	3	0	5	0	2	0	8
1959												
20 July	10	5	3	3	1	6	1	6	0	8	0	3
7 Aug.	6	4	2	8	0	10	0	12	0	7	0	6
2 Nov.	4	6	2	6	1	8	0	8	0	6	0	10
1960												
2 Jan.	0	30	0	8	0	6	0	5	0	8	—	—
Summary, excluding Jan. 1960	32	23	10	19	3	27	1	31	0	23	0	27

simultaneous maturity of male and female gonads, and despite the evidence for self-fertilization in other barnacle species (Barnes and Crisp, 1956), self-fertilization apparently does not take place in *M. polymerus*. It also appears that formation of ovigerous lamellae does not occur in the absence of fertilization.

DISCUSSION

On the subject of reproduction in cirripeds, a good deal has been written. Some accounts, such as those of Darwin (1851), Broch (1922), and others are concerned with the questions of hermaphroditism, the existence of complemental males, etc., rather than reproductive anatomy, reproductive cycles, and fecundity.

Comparison of the seasonal reproductive cycle of *M. polymerus* in Monterey Bay with that of *M. spinosus* on the New Zealand coast (Batham, 1944–45) shows some interesting features. Figure 4 summarizes reproductive and temperature data for the two species. In Monterey Bay, from November, 1956, through the greater part of April, 1957, shore temperatures remained between 11.2° C. and 12.4° C., but showed a definite rise toward 13° C. during February and March. From May, 1957, through about one-half of November, shore temperatures remained above 13° C., rising to over 14° C. in May, July, and August, and to above 16° C. during September and October. Thus, the year may be roughly divided into the colder winter months and the warmer spring, summer, and fall months.

In *M. polymerus*, the increase in mean seminal vesicle width during the early spring roughly parallels the rise in shore temperature; the decrease in mean seminal vesicle width in the winter follows roughly the winter decrease in temperature. Likewise, the earliest occurrence of embryos follows closely behind the seasonal rise in temperature, and embryos continue to be present in the population until just after temperatures begin to drop during the winter. There is thus a clear correspondence between reproduction and shore temperature. The three peaks seen

in the occurrence of embryos in the latter half of 1957, while not statistically significant, show a relationship with the temperature peaks of May, July, and September which is perhaps suggestive.

The data given by Batham (1944–45) for *M. spinosus,* from the open coast of Otago Peninsula, New Zealand, and the corresponding temperature curve (from two later years and a neighboring vicinity; Batham, 1958) show a similar relationship between temperature and reproductive activity. As might be expected for a related species occurring at a slightly higher latitude but in the southern hemisphere, the reproductive cycle is a rough mirror image of that of *M. polymerus* in Monterey Bay, California, though the breeding season is somewhat shorter.

Other barnacle species have been observed to be reproductively active primarily during the months of warmer water temperatures: *Balanus crenatus,* studied on the Atlantic coast of Canada (Bousfield, 1952–53) and in San Francisco Bay, California (Herz, 1933); *Balanus improvisus,* studied on the Atlantic coast of Canada (Bousfield, 1952–53); and *Chthamalus stellatus,* studied in Great Britain (Crisp, 1950).

While such a correlation between temperature and reproductive activity might seem obvious and only reasonable, a number of barnacles reproduce primarily when water temperatures are low or at a minimum. This group includes the following: *Balanus balanoides,* studied on the Atlantic coast of Canada (Bousfield, 1952–53), at Woods Hole, Massachusetts (Fish, 1925), and in Great Britain (Moore, 1935; Crisp and Patel, 1960); *Balanus hameri,* studied on the Atlantic coast of Canada (Bousfield, 1952–53), and in Great Britain (Crisp, 1954); *Balanus porcatus,* studied in Great Britain (Crisp, 1954); and *Balanus glandula,* studied at Vancouver, B. C., and La Jolla, California (Barnes and Barnes, 1956) and in San Francisco Bay, California (Herz, 1933).

There is evidence that still another group of barnacles reproduce (perhaps with some variation in rate) throughout the entire year. These include *Elminius modestus,* studied in Great Britain (Crisp and Davies, 1955), *Verruca stroemia,* studied in Great Britain (Pyefinch, 1948), and *Balanus tintinabulum,* observed at La Jolla, California (Coe, 1932).

Bousfield (1952–53) has studied the distribution and spawning seasons of the barnacles of the Atlantic coast of Canada, and reviewed the evidence supporting temperature as a principal factor governing reproduction in cirripeds. He clearly indicated that there is variability in reproductive period within a given species at different latitudes within its geographic range, and showed that reproductive activity tended to occur at times when temperatures were similar, regardless of latitude.

The relationship of temperature and food supply to rate of reproduction has been studied by Crisp and Davies (1955) in the barnacle *Elminius modestus.* By growing these barnacles on glass slides and observing development through the translucent bases, these workers were able to follow, *in vivo,* the development of both ovarian eggs and lamellar embryos. They found that (p. 379) "the time interval occupied by successive broods varies among individuals, and with the season of the year. Rate of development of embryos seems to be a function of temperature alone, but regeneration of the ovary depends on nutrition and food supply." Crisp (1959), working with *Balanus balanoides,* showed that the rates

of development of the early embryonic stages (through the limb bud stage) are temperature-dependent up to 12°–14° C., but that the later stages vary little in rate of development between 3° C. and 12° C.

Further points of comparison may be brought out between *M. polymerus*, *M. spinosus*, and other barnacles. The present study indicated that sexual maturity is not necessarily a function of size of the animals alone. Results of studies on *M. spinosus* and *Elminius modestus* showed similarly that in populations of smaller barnacles, sexually mature individuals are found, but less frequently than in populations of larger barnacles.

Self-fertilization apparently does not occur in large isolated individuals of *M. polymerus*, and cross-fertilization appears to be the rule. Crisp (1954) and Crisp and Patel (1960) pointed out that cross-fertilization also appears obligatory in *B. crenatus*, *Elminius modestus*, and in *B. balanoides*. However, self-fertilization very probably can occur in at least three species of acorn barnacles. Barnes and Crisp (1956) experimentally isolated individuals of *Chthamalus stellatus*, *Verruca stroemia*, and *Balanus perforatus* and found that they frequently produced ovigerous lamellae. They also observed that fertilized eggs found in such isolated individuals are frequently less viable than cross-fertilized eggs. The genetic advantage of cross-fertilization is well known, and it appears that the *Mitella polymerus* population, composed usually of closely-packed individuals, is well adapted for cross-fertilization.

Summary

1. The gross structure of the reproductive system of *M. polymerus* is described and compared with that of the southern hemisphere species, *M. spinosus* (studied by Batham, 1944–45).

2. Size and reproductive activity in *M. polymerus* are related. All animals over 27.5 mm. in breadth (distance from rostrum to carina) are found to reproduce; smaller animals are found to contain developing embryos less frequently. No sexually mature animals less than 17.2 mm. in breadth were found.

3. A fifteen-month study of the reproductive cycle in the population is described. Reproductive activity is evident during the greater part of the year. For the year of 1957, developing embryos were present in the population for a period of eight months during which time the shore temperature ranged from 12.3° C. to 17° C. The reproductive season for the southern hemisphere species likewise occurs during the warmest months; thus the yearly cycle of *M. polymerus* shows a perhaps expected mirror image of the situation occurring in the southern hemisphere.

4. Within individuals, male and female gonads mature at approximately the same time during the year, and during the greater part of the year, an individual contains both developing eggs and seminal vesicles full of sperm.

5. Stages of development of ovarian eggs and brooded embryos found simultaneously in individuals are compared. During the early and middle months of the reproductive season, ovarian eggs and brooded embryos tend to be in similar stages of development (that is, small eggs are found with early stage embryos, large eggs with late stage embryos, etc.). During the later reproductive months, a relative lag in ovarian egg development is evident.

6. Embryos were raised *in vitro* under controlled temperatures. The embryos took an average time of thirty days for development from fertilized egg to free-swimming larva.

7. Estimates are given of the number of broods of young and the number of larvae liberated by a large individual during a year, and these are compared with the results of Batham (1944–45) for *M. spinosus*. Studies of the larval brood period, the stages of eggs and embryos found simultaneously in individuals, and the length of the reproductive season allow an hypothesis that five to seven broods may be liberated by a large individual during a year. Three to four broods appears more probable for an average large animal. A pair of ovigerous lamellae may contain from 104,000 to 240,000 larvae. Batham's data (1944–45) showed that probably two broods, each containing approximately 3000 larvae, are liberated by *M. spinosus* during a year. Thus *M. polymerus* may liberate from 52 to 280 times as many larvae per year as a single large *M. spinosus*.

8. The possibility of self-fertilization in *M. polymerus* is studied. Relatively isolated large animals are found to carry ovigerous lamellae less frequently than those adjacent to each other; and large animals isolated from each other by over eight inches were never found carrying embryos. From this evidence, it appears that self-fertilization does not occur in this species, and that cross-fertilization is necessary for the formation of ovigerous lamellae.

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OBSERVATIONS ON THE NUTRITION OF THE RHYNCHOCOELAN LINEUS RUBER (O. F. MÜLLER)

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Feeding and digestion in the Rhynchocoela have received relatively little attention apart from brief accounts by Wilson (1900), Reisinger (1926), Coe (1943) and Hyman (1951). These indicate that the group is carnivorous, preying upon a variety of invertebrates which are captured by means of the extensible proboscis and swallowed whole, and that digestion may be either extracellular or partially intracellular. No further details of rhynchocoel nutrition are available and to remedy this deficiency, that of the common British species *Lineus ruber* (O. F. Müller) has been investigated.

Materials and Methods

Specimens of *Lineus ruber* were collected from beneath rocks embedded in sand at mid-tide level at Cremyll, Plymouth. After starvation for two days to induce a readiness to feed and to clear the gut of remnants of previous meals, individuals were presented with representatives of the fauna of their habitat, and the methods of capture and ingestion of the selected prey observed.

The course of digestion was followed by histological examination of individuals fixed at progressive intervals after feeding upon either the natural food or easily identifiable test foods such as frog erythrocytes and raw, optically active starch grains. Fixation was in Susa at 30° C. and sections cut at 8 μ were stained with the haematoxylin and eosin, Feulgen, periodic acid-Schiff (P.A.S.), Alcian blue (for mucin), benzidine-peroxide (for haemoglobin), and Lugol's iodine techniques. Changes in the pH of the gut contents during digestion were followed by feeding particles of fish muscle stained with 0.5% sea water solutions of various indicators and observing subsequent color changes by periodically flattening the fed individuals and examining by both reflected and transmitted light.

Food reserves were studied after fixation in Flemming (for fats) and 90% alcohol containing 1% picric acid (for carbohydrates and proteins). Sections of individuals fixed in the latter reagent were stained by the Best's carmine, P.A.S. and modified Millon's methods.

Observations

The food and feeding mechanism

Lineus ruber feeds mainly on small annelids and crustaceans but particles of any dead organic material will be taken, providing it is not too decomposed. The oligochaete *Clitellio arenarius* was particularly common in the habitat and at the time of collection (July–August) appeared to form the bulk of the food.

Living prey is detected by the eye-spots and a starved *Lineus* will respond to animals moving within 2 cm. of the head. Dead or injured animals emitting decomposition products or body fluids can be located at greater distances and here it is presumably chemoreceptors in the cephalic ciliated grooves which are stimulated. Detection of living prey is followed by immediate eversion of the proboscis [1] through the proboscis pore at the anterior tip of the body, and this occurs with such explosive force that as the proboscis strikes the prey it becomes coiled around it in a tight spiral grip. It does not penetrate the prey, since it lacks stylets or similar piercing organs, but the tightness of its grip may rupture the integument and cause loss of body fluids or gut contents. The grip is aided by sticky secretions from the proboscis epithelium and immediately it is secured the proboscis begins to retract and draws the prey, usually struggling violently, back towards the mouth. This lies ventrally 2–3 mm. behind the proboscis pore, and as the prey is drawn towards it the body anterior to the mouth is raised and extended until it can grasp the prey by curling downwards over it. This movement of the anterior tip of the body continues downwards and backwards and forces the prey into the mouth which gapes open to receive it. The proboscis then gradually relinquishes its grip and withdraws into the rhynchocoel as movements of the mouth, aided by contractions of the anterior body musculature, force the prey into the gut. Ingestion of small animals is completed in 15 to 20 seconds but with larger prey, such as annelids one-third to one-half the length of the feeding individual, it may take as long as 30 minutes, and in such cases the first part to be swallowed is partially disintegrated before ingestion is complete. Small animals usually die within seconds of entering the gut, but active errant polychaetes with armored jaws may survive long enough either to force their way to the exterior through the gut and body walls or propel themselves down the length of the gut to emerge unharmed at the anus. This is particularly liable to happen when the prey is ingested head first, but in the majority of cases the proboscis seizes an animal about its middle and consequently draws it back to the mouth bent upon itself in the shape of a U. It is then ingested in this form and is unable to escape from the gut before being killed.

During capture and ingestion *Lineus* extends to its fullest length and produces copious sticky mucoid secretions from the ventral surface. This enables a firm hold to be retained upon the substratum whilst dealing with the prey, and even if the latter is partially buried it can be drawn from its retreat and swallowed without causing the *Lineus* to shift position.

Inert masses of food, such as animal remains or the test foods used in this investigation, do not stimulate eversion of the proboscis but are seized directly by the mouth and swallowed piecemeal.

The structure of the gut

The gut consists of three histologically distinct regions, namely the mouth and buccal cavity, the foregut, and the intestine. It runs the length of the body from mouth to anus without coiling, is ciliated throughout and lacks both multicellular glands and musculature.

[1] Details of the proboscis and the mechanism of its eversion are given by Hyman (1951) and are not included here.

The mouth consists of a ventral subterminal invagination of the epidermis some 200 μ deep and opening directly into the buccal cavity. It is fringed with large cilia and externally has a lobed appearance due to folds in its walls which allow expansion during ingestion. The invaginated epidermis contains acidophil, P.A.S.- and Alcian blue-positive gland cells whose secretions probably facilitate passage of food, and the entire mouth region is surrounded by concentrations of similar sub-epidermal gland cells. The buccal cavity is lined by ciliated cuboidal cells 10–12 μ tall and these are backed by masses of acidophil and basophil gland cells, the majority of which stain with Alcian blue and appear to have the same function as those around the mouth. The walls of the cavity are much folded and ascend diagonally backwards to become continuous with those of the foregut beneath the proboscis sheath.

The foregut (Fig. 1) runs posteriorly for one-tenth the length of the animal and its walls are considerably thickened, especially ventrally where the wall may be up to 300 μ in depth. They are thrown into small simple folds and consist of a single layer of ciliated cuboidal cells, 10–12 μ tall, lining the lumen and lying upon acidophil syncytial tissue containing numerous gland cells, free nuclei and occasional large lacunae. In the anterior portion the gland cells consist of P.A.S.- and Alcian blue-positive acidophils and basophils in approximately equal amounts, together with a number of others which are intensely basophilic but give no reaction to Alcian blue. The proportion of the latter increases along the length of the foregut to the median portion where all the gland cells are of this type. The gut wall then gradually decreases in thickness and gland cell content as it nears the intestine and terminates in a constriction (Fig. 1) separating the latter from the foregut.

The intestine is the longest part of the gut and runs from its junction with the foregut in the anterior part of the body direct to the anus at the extreme posterior end. It bears paired and serially repeated lateral pouches or caeca throughout its length, apart from a short unpouched region immediately before the anus. The intestinal wall or gastrodermis (Fig. 3) is made up of two types of cells arranged in a single layer upon a thin basement membrane. The larger and more numerous of these are attenuated columnar cells, 50–55 μ tall and 8 μ wide, with granular basophilic cytoplasm containing various acidophil inclusions and basal vesicular nuclei. The free distal borders of the cells bear cilia which in unfed individuals are of uniform appearance and size, but in the presence of digesting food the cilia lose their uniformity and coalesce into pseudopodia-like processes which extend out into the lumen (Fig. 5). This peculiar modification of the cilia is correlated with entry of food material into the cells and is dealt with later.

The second type of cell found in the gastrodermis is glandular and occurs between the bases of the columnar cells. These gland cells (Figs. 2 and 3) are 40–50 μ tall and 5–6 μ wide, unciliated and contain up to thirty acidophil proteinaceous spheres, each 0.5 μ in diameter, which are discharged into the intestinal lumen when food enters from the foregut. They are most numerous in the anterior part of the intestine, where there may be as many as one gland cell to every three columnar, but this ratio is graded down the length of the intestine to approximately one in twenty in the middle region and one in fifty or more beyond until the gland cells disappear, finally, in the short unpouched region before the anus.

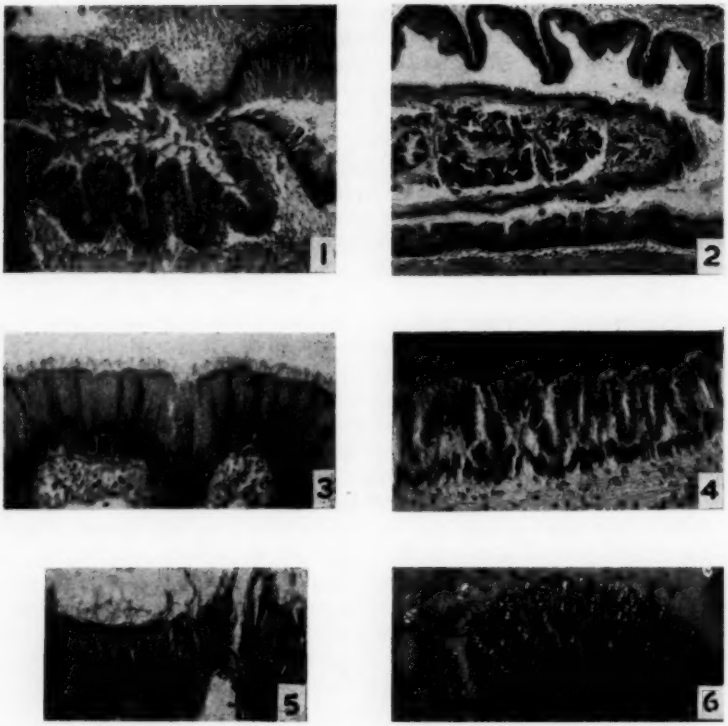

FIGURE 1. Longitudinal section of *Lineus* showing the posterior portion of the foregut (left) and the constriction which separates this from the intestine (right). Haematoxylin and eosin. Scale: 1 cm. = 50 μ.

FIGURE 2. Longitudinal section of the intestine in *Lineus* showing part of a newly ingested *Clitellio* lying intact and undamaged in the lumen. Acidophil gland cells are prominent in the gastrodermis in the lower portion of the figure. Haematoxylin and eosin. Scale: 1 cm. = 100 μ.

FIGURE 3. A portion of the gastrodermis in *Lineus* showing ciliated columnar cells interspersed with darker acidophil gland cells. Intestine empty. Haematoxylin and eosin. Scale: 1 cm. = 50 μ.

FIGURE 4. The gastrodermis in *Lineus* 30 minutes after a meal of frog erythrocytes. The intestinal lumen (top) contains a homogeneous mass of digested haemolyzed erythrocytes which stains heavily with Feulgen and almost obscures the ciliary processes. The columnar cells are loaded with engulfed spherical masses identical in nature with the contents of the lumen. Feulgen and light green. Scale: 1 cm. = 50 μ.

FIGURE 5. The gastrodermis in *Lineus* 30 minutes after a meal of raw starch grains. The cilia have coalesced into pseudopodia-like processes and a few starch grains already engulfed are ranged along the distal margins of the columnar cells. Lugol. Scale: 1 cm. = 50 μ.

FIGURE 6. The gastrodermis in *Lineus* 60 minutes after a meal of raw starch grains. The columnar cells are loaded with grains, many of which are as yet unchanged and still exhibit the characteristic black cross in polarized light. The lumen contains occasional free grains and on the left portions of two gregarine trophozoites with prominent nuclei. Section stained with haematoxylin and eosin and photographed by polarized light. Scale: 1 cm. = 40 μ.

The lateral pouches have the same structure as the rest of the intestine and are merely simple extensions to increase its area, not specialized digestive caeca.

The course of digestion

Ingested food passes rapidly through the buccal cavity into the foregut where it is held for a few seconds before its passage intact into the intestine (Fig. 2). Living food usually dies during the brief pause in the foregut and this is due, no doubt, to acid secretions from the numerous basophilic gland cells present here, for when particles of fish muscle stained with bromo-cresol purple or chlor-phenol red were fed, their pH value fell from 7.0 to 5.5 as they passed through the foregut, and sections of newly fed individuals showed the majority of the glands to be discharged.

There is no trituration or break-up of the food in the foregut but digestion begins immediately it enters the intestine. A series of individuals fixed at intervals after ingestion of the oligochaete *Clitellio* showed that as early as fifteen minutes after feeding the gland cells had discharged their spheres and digestion was well advanced. The *Clitellio* lay in the main median portion of the intestine with the epidermis deeply eroded and the entire body starting to fragment. The cilia of the columnar cells were still uniform in appearance and apparently creating currents in the gut contents to distribute the fragmenting food, for pieces of tissue were already passing into the lateral pouches. Digestion progressed rapidly with time and 30 minutes after feeding the intestine contained a heterogeneous mass of heavily eroded pieces of tissue, intact and fragmented setae, nephridia (which resisted digestion for longer than other tissues and stood out from these with surprising clarity) and diatoms, algal chains, etc. released from the oligochaete gut. The gastrodermal cilia were now beginning to lose their uniformity and coalesce into pseudopodia-like processes stretching into the lumen of the intestine, whilst semidigested material from the latter was appearing as acidophil spheres in the distal portions of the columnar cells bearing these structures. These spheres passed back deeper into the cells and their number increased rapidly with time. Sixty minutes after feeding, the material in the lumen was almost homogeneous, with setal fragments and diatom cases being the only recognizable elements in it, whilst the columnar cells of the gastrodermis were packed with spheres of food undergoing intracellular digestion. The spheres decreased in size and affinity for stains (especially the Millon reagent for protein) as they passed down the cells to disappear finally in the basal region, the cells presumably then passing the products of digestion to other tissues whilst taking up more semidigested material distally until the lumen was emptied. This occurred some six hours after feeding and the amount of intracellular material then rapidly decreased. After a further three hours the columnar cells contained only a few acidophil inclusions, their cilia had resumed their normal shape and size, and the gland cells were again full of enzymatic spheres. Indigestible residues were collected in the short unpouched region of the intestine near the anus, being swept there, probably, by the reconstituted cilia, and observations on living specimens showed that they were expelled eventually by a sudden contraction of the posterior body musculature.

A parallel series of feeding experiments, using fish muscle stained with indicators, showed that the initial drop in pH as the food passes through the foregut

is maintained in the intestine during digestion. In some cases it fell even further, to pH 5.0, and when sufficient indicator-stained material entered the columnar cells to be visible in neutral saline squashes, the intracellular digestion was seen to be progressing in a similarly acid medium of pH 5.0–5.5.

It was not clear from the *Clitellio*-fed series how semidigested material enters the columnar cells, but the pseudopodia-like appearance of the coalesced cilia and spherical compact form of the material when within the cells suggested a form of phagocytosis. This possibility was investigated by feeding *Lineus* on frog erythrocytes and raw, optically active starch grains made palatable by mixing with frog plasma, to ascertain whether such discrete particles were in fact taken into the columnar cells. In the series fed on erythrocytes, however, haemolysis occurred as they entered the intestine, the break-up including the majority of the nuclei, and 30 minutes after feeding the lumen contained a semidigested mass which gave a strong reaction with Feulgen, due to released nuclear materials, and with the benzidine-peroxide reaction for haemoglobin. The cilia had coalesced into the usual processes and many of the cells contained spherical masses with the same staining properties as the material in the lumen (Fig. 4). These apparently phagocytosed masses passed back into the cells as more appeared distally, and gradually decreased in size and their reaction to Feulgen and benzidine-peroxide as intracellular digestion progressed. Digestion of the blood meal was completed in six hours and the intracellular spheres disappeared without leaving residues of haematin or other insoluble pigments from the degradation of the haemoglobin.

Final confirmation of the occurrence of phagocytosis came from the series fed on starch grains. Thirty minutes after feeding the cilia had formed filamentous processes extending into the lumen, and a few intact grains, staining blue with Lugol and still exhibiting the characteristic black cross in polarized light, had already been taken into the cells and were ranged along their distal margins (Fig. 5). The number of such grains increased rapidly and 60 minutes after feeding packed the columnar cells (Fig. 6). Only grains 5 μ or less in diameter were engulfed and larger ones remained in the lumen where they gradually lost their optical activity, fragmented and stained brown with Lugol. The fragments then passed into the cells and joined the previously engulfed grains which were undergoing intracellular digestion, losing their identity and disappearing towards the bases of the cells.

Parasites of the gut

Approximately 75% of the *Lineus* examined contained in the intestine an acephaline eugregarine identified as *Urospora nemertes* (Koelliker). Trophozoites (Fig. 6) 150–180 μ long and 15–20 μ wide, with basophil, P.A.S.-positive cytoplasm and prominent nuclei, occurred in all parts of the lumen, and the intracellular stages, strikingly prominent with P.A.S., were common in the columnar cells. The gregarine did not appear to harm *Lineus* in any way, apart from a few occasions when infected columnar cells reacted against developing intracellular stages and caused them to degenerate into masses of yellowish brown crystals. Such cells then burst, either *in situ* or after being shed into the lumen, and the crystals were eliminated with the faeces.

The food reserves

Fat forms the principal food reserve in *Lineus* and occurs as intracellular globules up to 5 μ in diameter in the parenchyma and, to a lesser extent, in the columnar cells of the intestine. There are no specific protein reserves and the only demonstrable carbohydrate reserve is in the form of tiny granules of glycogen scattered throughout the parenchyma, musculature, and columnar cells.

Discussion

The main points of interest in the nutrition of *Lineus ruber* lie in the feeding mechanism and the digestive processes. In the case of the former a simple but effective method of capturing the food, supplemented by slight modification of the anterior portion of the alimentary canal into a thick-walled glandular foregut for its reception and killing, enables this rhynchocoelan to prey successfully upon animals far more active and elaborate than itself. In this respect *Lineus* resembles the turbellarian flatworms where similarly simple feeding mechanisms make available prey ranging from protozoa to molluscs and tunicates (Jennings, 1957; 1959a). In the Turbellaria it is the pharynx which forms the principal element of the feeding mechanism and this organ is thus analogous in function to the rhynchocoelan proboscis as seen in *Lineus*. The only disadvantage apparent in the type of feeding found in *Lineus* is the possibility of escape by the prey before the secretions of the foregut can take effect but this is overcome, no doubt, in those rhynchocoelans which possess a proboscis armed with stylets and poison glands by killing or paralyzing the prey at the moment of capture.

Digestion in *Lineus* follows a pattern observed in other Acoelomata (Jennings, 1957; 1959b) in that both extracellular and intracellular processes are concerned, but here the intestinal wall is ciliated and consequently the semidigested food would be expected to enter by absorption. In fact, however, it enters by a form of phagocytosis, as is proved beyond doubt by the appearance in the columnar cells of starch grains which retain their form and optical activity after entry and so must have passed into the cells as solid discrete particles. This method of taking material into the columnar cells involves temporary modifications in the form and behavior of the cilia during the digestion of each meal, and the protoplasmic pseudopodia-like processes formed from the coalescence of neighboring cilia are probably concerned in the engulfing of semidigested food, although this has not been observed histologically. The need for the intestine to be ciliated probably arises from its length and the absence of musculature, which together create a need for some method of distributing fragmenting food in the early stages of digestion and collecting residues near the anus at the end. Contractions of the body musculature appear to be insufficient for anything but the final expulsion of the collected residues and hence ciliary currents are used. The reason for the retention of phagocytic uptake of food under these conditions is unknown, for there is no apparent reason why extracellular digestion should not be carried to a point where the semidigested food is soluble enough for absorption, and this presents an interesting subject for further investigations.

SUMMARY

1. The rhynchocoelan *Lineus ruber* feeds on small annelids and crustaceans which are captured by the unarmed proboscis and swallowed whole.

2. The alimentary canal is differentiated into three regions: a buccal cavity, a foregut where the prey is killed by acid secretions, and an intestine where it is digested.

3. Digestion is the result of both extracellular and intracellular processes and occurs in an acid medium of pH 5.0–5.5. The enzymes responsible for the initial extracellular breakdown come from gland cells in the intestinal wall and digestion is completed within the columnar cells of the latter. Semidigested food enters these columnar cells by a phagocytic process and this involves temporary modifications in the form and function of their cilia.

4. The food reserves consist of fat deposits in the parenchyma and, to a lesser extent, in the columnar cells of the intestine.

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HERMAPHRODITISM IN THE SEA SCALLOP, PLACOPECTEN MAGELLANICUS (GMELIN)

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While hermaphroditism is of common occurrence among normally dioecious mollusks, a careful search of the literature failed to reveal any published record of this condition for the commercially important sea scallop, *Placopecten magellanicus*. Consequently, observations on the occurrence of the phenomenon in this species are of some interest and importance.

Our first hermaphroditic sea scallop was collected on September 17, 1959, while doing routine investigatory work relative to the scallop fishery aboard the chartered scalloper *Whaling City* in the western part of Georges Bank (68° 45′ W. Long., 43° 03′ N. Lat.). September is within the spawning season for the sea scallop on Georges Bank (Posgay and Norman, 1958). However, spawning had not yet started at this locality and all specimens, including the hermaphrodite, were ripe and full.

After close macroscopic examination the hermaphroditic gland was fixed for histological study in Newcomer's (1953) fluid and stained either by means of the Feulgen reaction or with Harris' hematoxylin and eosin. Some Feulgen-stained sections were counterstained with light green (yellowish). Pieces of tissue were cleared in chloroform, embedded in paraffin, and sectioned at 10 micra. For comparison normal male and female gonads were likewise treated except that in these Bouin's fluid was the fixative.

In the normal gonad the size and shape are similar in both sexes. The single gonad is tongue-shaped and occupies most of that portion of the body ventral to the foot. It extends dorsally to form a thin layer over the surface of a portion of the digestive gland. The genital organ is large and plump when ripe but after spawning it becomes much smaller, shriveled, and quite flaccid. The sexual products are easily seen through the tissue of the gonads, the ova giving the female gonad a bright coral red appearance at maturity, the sperm producing a whitish-cream coloration in the male gonad.

The hermaphrodite mentioned above has the male and female parts located in different regions of the same gonad. The proximal part forms the ovary while the testis lies distal to it. The boundary between the two regions is indefinite with quite irregular islets of one tissue occurring within the tissue of the other (Fig. 1a). This is even more pronounced histologically (Fig. 2a). The gonad is unspent and, relative to the degree of development, compares favorably with normal unspent gonads.

[1] Grant 2E-41, National Institute of Allergy and Infectious Diseases, U. S. Public Health Service (in part).

The ovarian follicles are closely crowded and are filled with mature ova which take the shape of polyhedrons because of their tightly packed condition. The lumina of the follicles are completely filled. Each follicle is lined on the outside by a single cell layer of squamous epithelium. Inside the follicles the individual ova are separated by a non-granular intracellular substance sandwiched between and separating the cell membranes. This matrix is rather uniformly distributed and of little variation in thickness. Actually, it may not be intracellular in the strict sense but a component of the cell membranes. Mature ova average about 45–50 micra in diameter in fixed material. Their germinal vesicles are large and clear, and contain a fine network of chromatin and several conspicuous nucleoli (Fig. 2b). Connective tissue or follicular cells are rarely found between either male or female follicles in this individual.

The testicular follicles are tightly packed and are covered by the same kind of squamous cell layer as found covering the ovarian follicles. Inside the follicle and adjacent to the covering epithelium is a layer of spermatogonial cells. This layer of primordial germ cells is one to several cells thick (usually two or three, but sometimes up to ten or more). Progressing inward toward the lumen of the follicle are the primary and secondary spermatocytes, followed by spermatids, then mature sperm at or near the center. The tails of the spermatids are usually located in the most central part of the follicle. In portions of the testis where spermato-

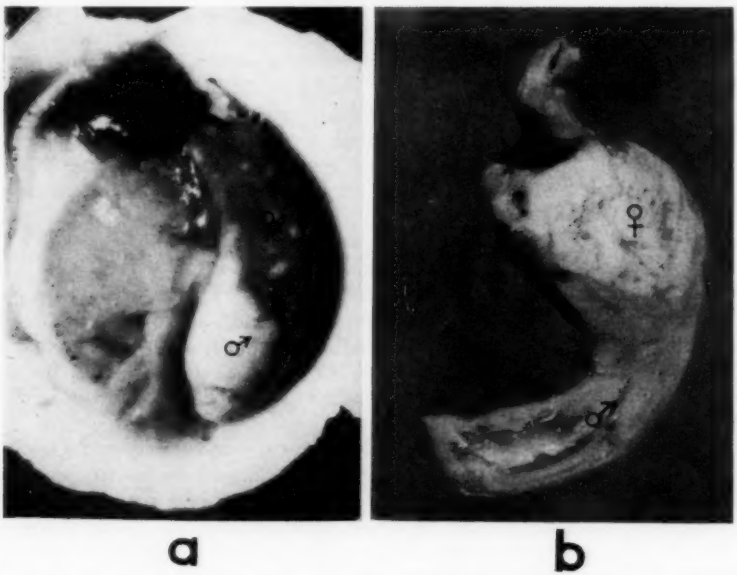

FIGURE 1a. Hermaphroditic *Placopecten magellanicus*. Mantle and gill tissue folded back to show the large bisexual gonad (× 2/3). 1b. Hermaphroditic gonad of *P. magellanicus*. Male portion has spawned, female (bulbous) part still unspawned (× 2). (Note: gonad fixed in Bouin's and preserved in alcohol before photo taken, this resulting in loss of color.)

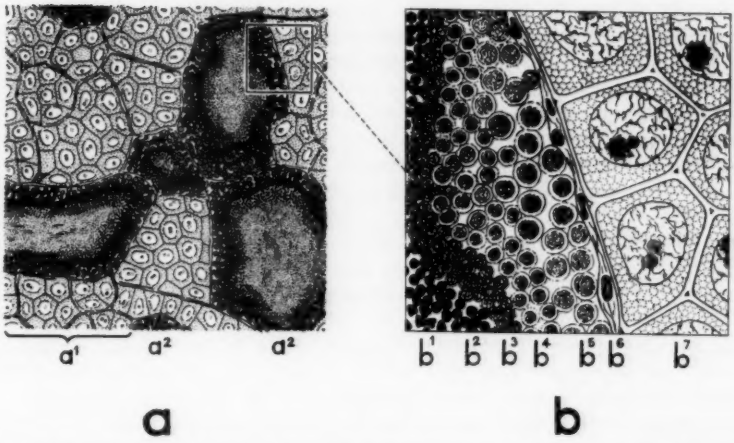

FIGURE 2a. Histological section through central portion of the hermaphroditic gland of specimen shown in Figure 1a (× 50). a^1, Portions of female follicles filled with ova. a^2, Portions of male follicles containing all stages of spermatogenesis. 2b. Enlarged portion of Figure 2a showing part of a male and a female follicle and their boundary (× 250). b^1, Mature sperm. b^2, Spermatids. b^3, Secondary spermatocytes. b^4, Primary spermatocytes. b^5, Spermatogonia. b^6, Epithelial lining. b^7, Portion of a mature ovum.

genesis is completed, the follicles are completely filled, from epithelial lining to epithelial lining, with mature sperm. However, even in such follicles, there are occasional cells at the epithelium which we take to be primordial germ cells but which may perhaps be follicle cells.

The number of spermatogonial divisions before synapsis can not be determined, but there is no evidence in this specimen, or in normal unisexual males, to suggest that there are not more than two (*e.g.*, see Coe and Turner, 1938, Fig. 17, p. 103). Since whole bunches of spermatogonia, ten or more cells deep, are sometimes seen to project out toward the lumen of the follicle, they probably go through a series of divisions before synapsis. In pulmonate gastropods, where the number of spermatogonial divisions can be accurately determined, there are normally six such divisions prior to spermatocyte formation (Burch, 1959).

Spermatogonia average about 5 micra in diameter in sectioned material, primary spermatocytes about 2.5 micra in diameter, and the width of each sperm head is about 1.7 micra.

The individual follicles in this hermaphroditic gland are either all male or all female; there are no ambisexual follicles. In the proximal end of the gland most follicles are female, and in the distal end most are male. But, in a wide central area both male and female follicles are widely and indiscriminately dispersed (Fig. 2a). The size and appearance of the cells, as far as can be ascertained, are identical to similar stages in normal individuals of this species. Atypical spermatogenesis as found by Coe and Turner (1938) was not observed.

A second hermaphrodite was found on November 21, 1959, in a sample from the eastern part of Georges Bank (66° 45′ W. Long., 41° 23′ N. Lat.). The

arrangement of gonadal tissue in this specimen was similar to that of the first. However, the spermary was mostly spent while the ovary still remained large and plump (Fig. 1b) with no sign of having started to spawn. This differs from the observed spawning habit of the normally monoecious great scallop, *Pecten maximus*. In this species the products are released within a few hours of each other, with either the eggs or the sperm being shed first (Mason, 1958). Also, the arrangement of the genital tissues within the gonad of *P. maximus* is the reverse of that of the two hermaphroditic *P. magellanicus* (*e.g.*, the testis proximal and the ovary distal).

Of colleagues who have investigated various aspects of the scallop fishery, only Dickie[2] (personal communication) has advised of having observed this phenomenon. He remembered seeing hermaphroditic sea scallops on two separate occasions. Once during the summer of 1956 he was shown several preserved mature hermaphroditic gonads from Georges Bank by one of the scallop-vessel skippers. Another time (July 22, 1949) he found a 95-mm. predominantly female hermaphrodite from "Hour Ground" off Digby, Nova Scotia.

Hermaphroditism is the usual condition in most of the Pectinidae (Coe, 1945); *P. magellanicus* is an exception. Hermaphrodites in other unisexual genera of pelecypods are not uncommon and it seems likely that the occasional deviations in the developmental processes which produce these hermaphrodites are due to the failure of the sex-differentiating mechanism to function normally, as has been suggested by Coe and others. According to the classification of Coe (1942) these abnormalities would be termed *accidental functional ambisexuals*, in which the primary sex factors go astray and fail to activate or suppress either the male or female hereditary mechanism at some early stage of development. This results in various amounts of both kinds of tissue being produced.

Young's (1941) suggestion that hermaphroditism in normally bisexual species may be due to aberrant chromosomal behavior during gametogenesis has not yet been corroborated by cytological or experimental evidence. Similar suppositions were advanced to account for production of male and female cells in the ovotestis of the pulmonate gastropod *Lymnaea stagnalis appressa* (= *L. s. jugularis*) by Crabb (1927) and in *Physa gyrina* by Mahoney (1940). Perrot (1930), however, showed that this was not the case for *L. stagnalis* and Burch and Bush (1960) have shown the observations on *P. gyrina* to be in error.

In the commercially important bivalves in which sex has been extensively studied, and which are dioecious, occasional occurrence of hermaphrodites is normal. Thus, to list a few examples common to the North Atlantic coastline, Thorson (1936) remarked that *Mytilus edulis* has a considerable percentage of hermaphrodites. Loosanoff (1936) in his sexual studies of the quahog (*Mercenaria mercenaria*) found two cases of functional hermaphroditism among several hundred mature clams. Also, Turner[3] (personal communication) mentioned having seen a functional hermaphrodite in this species. He observed it to release the sperm first, and then the eggs. Coe and Turner (1938) found three cases of hermaphroditism on examining about a thousand soft-shelled clams (*Mya arenaria*). In the case of *P. magellanicus* about 3000 gonads were inspected after the first and

[2] Lloyd M. Dickie, Biologist, Biological Station, St. Andrews, New Brunswick, Canada.
[3] Harry J. Turner, Jr., Marine Biologist, Woods Hole Oceanographic Institution.

before the second hermaphrodite was found. The low frequency of occurrence accounts for this condition seldom being observed in the sea scallop.

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ANTIGENS OF ARBACIA SPERM EXTRACTS[1]

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The initial steps in fertilization appear to involve interactions of the sperm and egg surfaces at the molecular level (see Tyler, 1948, 1949; Metz, 1957a, 1957b). Most of the present information concerning such interaction has been obtained from studies of egg and sperm extracts. Among sperm extracts those with action on eggs have commanded most interest. In the sea urchin and certain other forms, extracts prepared by a variety of methods precipitate the egg jelly layer, agglutinate eggs and neutralize the sperm agglutinating action of the fertilizin obtained from eggs. These effects of the extracts may result from the action of the sperm-surface receptor substance, antifertilizin, with which fertilizin combines in the sperm agglutination reaction (*e.g.* Tyler, 1948; Metz, 1957b). Whether or not these effects are to be identified with antifertilizin, absorption experiments have shown that such extracts contain some antigens in common with those of the sperm surface (Köhler and Metz, 1959a, 1959b, 1960). Further examination to reveal a more complete spectrum of antigens in such extracts seemed desirable. Accordingly, in the present investigation, *Arbacia* sperm extracts were examined for antigens by means of agar diffusion and immunoelectrophoretic techniques. The study revealed a maximum of four antigens in extracts prepared by freeze-thawing sperm.

Material and Methods

Arbacia punctulata from the vicinity of the Florida State University Marine Laboratory, Alligator Point, Florida, and from Woods Hole, Massachusetts, were used in the study. Semen was usually obtained from the animals by electrical stimulation (Harvey, 1956). The spermatozoa were separated from the seminal plasma by centrifugation (approximately $3000 \times g$; 20 minutes) at 4° C. The packed sperm were resuspended once in sea water and settled again by low speed centrifugation. The final supernatants following such washing regularly failed to give precipitation bands when diffused against anti-sperm serum. Standard sperm suspensions were prepared by diluting the packed sperm with three volumes of sea water.

Sperm extracts were prepared from such suspensions of washed sperm by a variety of methods. These included the established methods for preparing agents

[1] Contribution from the Oceanographic Institute, Florida State University. Aided by grants from the National Science Foundation, the National Institutes of Health and the Research Council of Florida State University.

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which act upon the egg jelly layer, namely, heating sperm to 100° C. (Frank, 1939) and freeze-thawing sperm (Tyler, 1939). The latter extracts are called "frozen-thawed extracts" below. Other methods are described with the individual experiments.

Antisera were prepared by injecting rabbits with sperm (25% washed sperm in sea water). The immunizing antigens were administered through intravenous, intraperitoneal and subscapular injections. In the last instance the antigen was injected as an emulsion in Freund's adjuvant (Difco). Several anti-*Arbacia* sera were examined. With the exceptions noted in the text the experiments reported here were performed with serum from the hyper-immune rabbit "#33." This rabbit received three injections of antigen in Freund's adjuvant (Difco) over a period of five months and was bled two weeks subsequent to the final injection. The immune serum obtained regularly agglutinated sperm to dilutions of 2^{-8} to 2^{-10}. No sera were pooled.

Agar diffusion and electrophoresis. Agar diffusion experiments using the technique of Ouchterlony (1948) were performed in 2% agar containing Merthiolate (0.01%) as a preservative. The reaction plates were incubated at room temperature for several weeks. Immunoelectrophoretic analysis (Wunderly, 1957) was performed using 2% agar blocks prepared in 0.05 ionic strength veronal buffer, pH 8.5 and containing 0.01% Merthiolate. Wells in the agar block were filled with antigen prepared as follows: after dialysis against 0.05 ionic strength veronal, the antigen was heated to 45° C. and mixed with an equal volume of melted (45° C.) 4% agar, also in veronal buffer. The mixture was then pipetted into the antigen wells of the agar slab and allowed to solidify. Agar slabs measuring approximately 20 × 6.05 cm. with antigen wells of 0.3–0.4 ml. capacity were used in these experiments. To achieve electrophoretic migration the preparations were subjected to a current of 25 ma for about six hours.

To improve the resolution of precipitin bands the agar blocks were fixed in 2% acetic acid, stained with Amidoschwarz (0.1% in acetate buffer, pH 4.0 solution) and destained in methanol-water-acetic acid (45:45:10).

Results

Agar diffusion precipitin tests were performed on extracts prepared by freeze-thawing washed *Arbacia* sperm and subsequently centrifuging the extracts in a clinical centrifuge (approximately 3000 × *g*). When diffused against anti-whole sperm serum such extracts yielded a maximum of four precipitin bands. Proceeding from the antigen to the antibody well in the agar plate, these four bands are designated a, b, c, and d. As seen in Figure 1, some variation in band number was found in repeated tests (*e.g.* two bands in Figures 1a5, 1b2, 1b3; three bands in 1b4, 1c6; four bands in 1c1, 1d3). Antisera other than #33 gave one to two bands. These differences in tests with different "frozen-thawed extracts" using serum #33 are attributed to differences in antigen concentration. However, the possibility of qualitative differences has not been eliminated. Differences in the band number using other antisera may reflect differences in concentration of antibody as well as antigen in the tests. In view of the fact that the sperm were washed sufficiently before extraction to remove seminal plasma antigens, the pre-

cipitin bands in the extracts must have arisen from antigens extracted from the sperm cells.

In this connection it is of interest that undiluted seminal plasma forms three bands when diffused against anti-whole sperm serum (Fig. 1a4, 1b1). Two of these seminal plasma bands join but do not cross two of the "frozen-thawed extract" bands. It appears, then, that the seminal plasma shares at least two antigens with the extract. In fact these seminal plasma antigens may have diffused from the

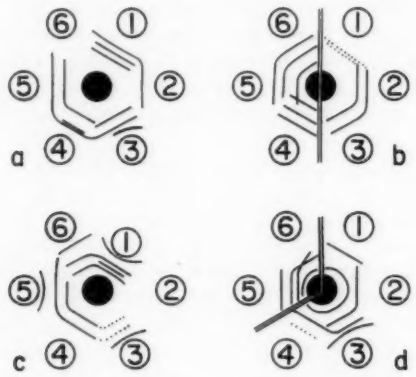

FIGURE 1. Ouchterlony agar diffusion tests. The center wells of all plates contain anti-*Arbacia* sperm serum No. 33. All surrounding wells were filled with extract and fluid of *Arbacia punctulata*. Each well received 0.5 ml. of the sample: (a) (1) supernatant over frozen-thawed sperm after centrifugation at 26,000 × *g* for 20 minutes; (2) residue of No. (1), resuspended in sea water and centrifuged at low speed; (3) supernatant over Mickle-disintegrated sperm; (4) seminal plasma; (5) "frozen-thawed extract" of sperm, low speed centrifuged; (6) body fluid; (b) (1) seminal plasma; (2) "frozen-thawed extract" of whole sperm, low speed centrifugation; (3) "frozen-thawed extract" of whole sperm, low speed centrifugation; (4) "frozen-thawed extract," low speed centrifugation; (5) supernatant over Mickle-disintegrated sperm, low speed centrifugation; (6) supernatant over Mickle-disintegrated sperm, low speed centrifugation; (c) (1) "frozen-thawed extract," low speed centrifugation; (2) basic protein, pH 0.9 extract; (3) supernatant over washed sperm after standing (aging), low speed centrifugation; (4) supernatant over citric acid-extracted sperm, low speed centrifugation; (5) supernatant over urea-treated sperm; (6) frozen-thawed extract, low speed centrifugation; (d) (1) isolated heads, "frozen-thawed extract," low speed centrifugation; (2) isolated tails, "frozen-thawed extract," low speed centrifugation; (3) whole sperm, "frozen-thawed extract," low speed centrifugation; (4) isolated heads, "frozen-thawed extract," low speed centrifugation; (5) acid extract (pH 3) of sperm, low speed centrifugation; (6) acid extract (pH 1.9) of sperm, low speed centrifugation.

sperm. However, the one band that has been clearly demonstrated in the supernatant of aging sperm is band *a* of "frozen-thawed extract" (Fig. 1c3). It should be noted that the three bands just described do not constitute the complete antigenic spectrum of *Arbacia* seminal plasma. As seen in Figure 2d, immunoelectrophoresis resolved seven bands in this material. Three of these correspond in position to three precipitin bands in the sperm extract (Fig. 2c).

It should be clear from these results that extracts prepared by freeze-thawing

sperm are not solutions of a single macromolecule. On the assumption that each precipitin band represents a single antigen, the extracts can contain up to four different antigens. Extracts of sperm prepared by other procedures also contained antigens. As seen in Figure 1d5, extraction of *Arbacia* sperm at pH 3 yielded a preparation which produced four precipitin bands. Evidently two of these antigens are labile to or are insoluble in stronger acids for, as seen in Figure 1d6, an aliquot of the same sperm suspension extracted at pH 1.9 yielded but two precipitin bands. Extraction at even lower pH (pH 0.9) yielded preparations that failed to form precipitin bands. The extracts prepared by extracting *Arbacia* sperm at pH 3 failed to precipitate egg jellies.

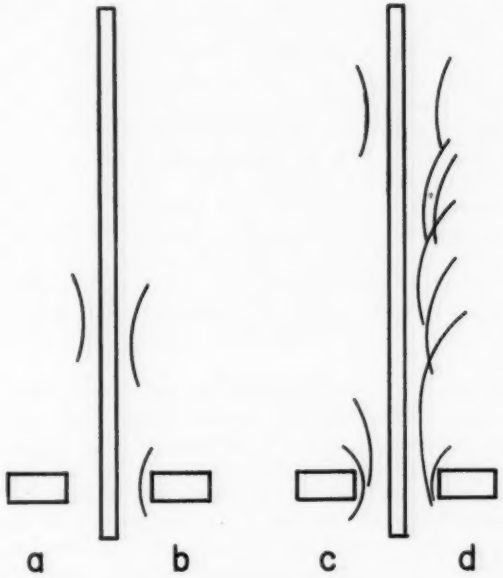

FIGURE 2. Immunoelectrophoresis in agar of various fluids from *Arbacia*. The central channel contains anti-*Arbacia* sperm serum No. 33, lateral channels contain preinjection serum No. 33. (a) Heated sperm extract; (b) "frozen-thawed extract," low speed centrifugation; (c) "frozen-thawed extract," low speed centrifugation; (d) seminal plasma.

Likewise, extracts prepared by treatment of sperm in the Mickle disintegrator (20 minutes, 20° C.) yielded three precipitin bands (Fig. 1b5 and 1b6). These have not been homologized with the antigens of "frozen-thawed extract." However, two (Fig. 1b4 and 1b5) of the bands correspond in position to two of the bands from "frozen-thawed extract." The third band crosses one of the bands of the "frozen-thawed extract" (probably the *d* band) and may represent an antigen not present in "frozen-thawed extract." Other extracting agents used were 1/18 *M* Na-citrate and 4 *M* urea. As seen in Figures 1c4 and 1c5, citrate extract pro-

duced two bands and urea extract three. The two citrate extract bands join two of the three urea extract bands. The third band of the urea extract is probably identical with the *a* band of "frozen-thawed extract."

As might be expected, extracts prepared by heating (100° C.) *Arbacia* sperm for one to five minutes do not have the full complement of soluble antigens. Such extracts at most yield a single band when diffused against anti-whole sperm serum. This antigen is evidently present in low concentration in extracts prepared by heating 25% sperm suspensions, for most such preparations fail to produce any precipitin bands in Ouchterlony or immunoelectrophoretic tests (*e.g.* Fig. 3a3). The band that does appear corresponds in position to one of the immunoelectrophoresis bands of "frozen-thawed extract" (see Figure 2 and below).

As seen above, extracts of whole sperm can contain at least four precipitating antigens. As in the case of the sperm agglutination antigens (Köhler and Metz, 1960), it seemed of interest to attempt to determine if these are present in both sperm heads and tails. Accordingly, sperm were broken into heads and tails by Mickle disintegration, these structures were separated by differential centrifugation and finally extracted by freeze-thawing (for details, see Köhler and Metz, 1960). When examined for precipitating antigens by agar diffusion, the frozen-thawed whole sperm extract produced four bands (Fig. 1d3). Head and tail extracts, adjusted to corresponding concentration, each produced two bands. One of these was common to both extracts and joined the *d* band of whole sperm extract. The second band in the head and tail extracts also joined bands produced by whole sperm extracts. However, these bands were not (Fig. 1d4) identical. The second band from the head extract joined the *b* band of whole sperm extract whereas the second band of tail extract joined the *c* band of the whole sperm extract (Fig. 1d2).

From the foregoing, it appears that both head and tail extracts lack band *a* of whole sperm extract. The absence of band *a* in the head and tail extracts is attributed to loss of this readily extracted antigen in the isolation process. Band *b* is present in extracts of isolated heads, but not tails; band *c* is present in tails but not heads and band *d* is present in both head and tail extracts.

Immunoelectrophoresis. In attempts to further resolve the antigenic composition of sperm extracts and seminal plasma these were subjected to immunoelectrophoresis in agar blocks (2% agar in 0.05 ionic strength veronal, pH 8.6). This method resolved three precipitin arcs (in three separate experiments) in frozen-thawed sperm extracts. As seen in Figure 2, b and c, one of these moved rapidly toward the anode, a second moved with intermediate speed and the third did not move appreciably. In comparative studies seminal plasma produced seven definite arcs (Fig. 2d). Three of these corresponded in position to the three arcs of the frozen-thawed sperm extract. Heat-extracted sperm (100° C., one minute) never produced more than a single arc (Fig. 2a). This corresponded in position to the fastest migrating antigen of unheated extract and seminal plasma.

Experiments with extracts centrifuged at high speed. In the studies presented above (Figs. 1 and 2), the extracts of frozen-thawed sperm were centrifuged at low speed (*e.g.* $3000 \times g$) only. Upon high speed centrifugation (*e.g.* $26{,}000 \times g$) of such extracts a pink, semi-gelatinous pellet is obtained and much, if not all of the egg jelly precipitating activity of the extracts is associated with this sedi-

mentable material (Köhler and Metz, 1959b). Therefore, a comparative study of extracts before and after such high speed centrifugation was undertaken. This showed that three of the bands (b, c, d) remained in the supernatant of "frozen-thawed extract" after centrifugation at 26,000 × *g* (Fig. 3a1 and 3a4). The *a* band was absent from such preparations. The pink pellet obtained after the high speed centrifugation was homogenized in a small amount of sea water, and gave only the *a* band when diffused against anti-sperm serum (Fig. 3a2 and 3a6). This result suggests that the *a* band (e.g. in Fig. 1a3, 1c1, 1c3 and 1d3) represents antigenic material, that is readily sedimented by high speed centrifugation.

In comparative studies with extracts prepared by other means and centrifuged at high speed, frozen-thawed and acid extracts (pH 3.2) showed one band (probably the *c* band) in common (Fig. 3a5). In a second experiment (Fig. 3c and 3d) only two bands were resolved in the high speed supernatant of "frozen-thawed extract." One of these (probably the *b* band) is common to the high speed supernatants of seminal plasma (Fig. 3c3) and heat extract (Fig. 3c6) and the low speed sediment of the original "frozen-thawed extract." Histone prepared by pH 0.8 extraction of sperm and nucleoprotein prepared according to Mirsky and Pollister (1942) gave no precipitation bands with the antiserum after high speed centrifugation (Fig. 3d). This is not surprising since histone and nucleoprotein are generally found to be poor antigens (Cushing and Campbell, 1957).

The supernatant obtained after high speed centrifugation of "frozen-thawed extracts" was also compared with extracts obtained by aging sperm (48 hours, in sea water), extraction with 4 *M* urea and with 1/18 *M* citrate for 48 hours (Fig. 3b). One band, possibly the *c* band, is common to all of these extracts.

Discussion

The results reported here show that extracts of frozen-thawed *Arbacia* sperm can yield four distinct precipitation bands when diffused against anti-whole sperm serum. These four bands are interpreted as four separate antigens. Detection of all of these antigens in agar diffusion precipitin tests appears to depend upon having high titer antisera and concentrated solutions of extract. Indeed these antigens may be only slightly soluble substances. Other extraction procedures (*e.g.* heating, acid, citric acid, and urea extraction) have not clearly revealed additional antigens. Likewise immunoelectrophoretic analysis revealed three antigens in frozen-thawed extracts. It appears, then, that only a few sea water-soluble antigens are obtained from *Arbacia* sperm in appreciable concentration. Possibly immunization of additional rabbits or other animals might yield an antiserum of unusual resolving power and reveal additional sperm antigens. However, the experience so far suggests that but few additional antigens would be discovered. The relatively small number of antigens in sperm extracts is in sharp contrast to *Arbacia* seminal plasma and egg extracts prepared by freeze-thawing. Parallel immunoelectrophoretic studies using anti-*Arbacia* egg sera and frozen-thawed extracts of *Arbacia* eggs readily resolved nine antigens. Likewise Perlmann (1953) found at least ten precipitin bands in agar diffusion tests using 0.15 *M* NaCl extracts of *Paracentrotus lividus* eggs. In preliminary tests no cross precipitin reactions were obtained between sperm extracts and anti-egg sera or the reverse.

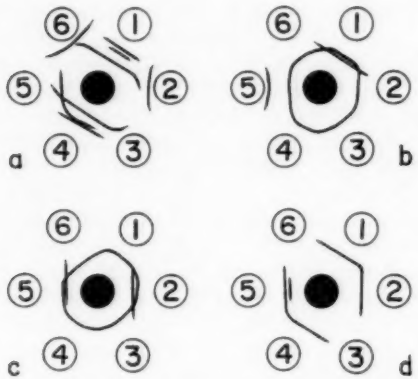

FIGURE 3. Ouchterlony agar diffusion tests. The center wells of all plates contain anti-*Arbacia* sperm serum No. 33. All surrounding wells were filled with extracts or fluids of *Arbacia punctulata*. Each well received 0.5 ml. of sample: (a) (1) "frozen-thawed extract" immediately subjected to centrifugation at 26,000 × *g*; (2) the pellet from No. 1 and No. 4, washed and resuspended; (3) heat extract, centrifuged at 26,000 × *g*; (4) "frozen-thawed extract" immediately subjected to centrifugation at 26,000 × *g*; (5) acid extract, centrifuged at 26,000 × *g*; (6) the pellet from No. 1 and No. 4, washed and resuspended. (b) (1) A very active egg jelly precipitating preparation obtained by resuspending the cake of frozen-thawed sperm followed by centrifugation at low speed; (2) 4 *M* urea extract of sperm, centrifuged at 26,000 × *g*; (3) 1/18 *M* Na-citrate extract of sperm, centrifuged at 26,000 × *g*; (4) supernatant over aged sperm centrifuged at 26,000 × *g*; (5) 4 *M* urea extract of sperm centrifuged at 26,000 × *g*; (6) 1/18 *M* Na-citrate extract of sperm, centrifuged at 26,000 × *g*. (c) (1) and (4) Supernatant over frozen-thawed sperm after high speed centrifugation; (2) and (5) the cake of frozen-thawed sperm was resuspended (same as Figure 3b1) in sea water for additional extraction (wells contain the supernatant after low speed centrifugation); (3) seminal plasma centrifuged at 26,000 × *g*; (6) heat extract, centrifuged at 26,000 × *g*. (d) (1) and (4) Supernatant over frozen-thawed sperm after high speed centrifugation; (2) and (5) the cake of frozen-thawed sperm was resuspended (same as Figure 3b1) in sea water for additional extraction (wells contain the supernatant after low speed centrifugation); (3) nucleoprotein centrifuged at 26,000 × *g*; (6) histone centrifuged at 26,000 × *g*.

Perlmann (1953), however, obtained one precipitin band when saline (0.14 *M*) extract of sperm was diffused against anti-egg serum.

The relationship, if any, of the four soluble sperm antigens to the sperm surface and to the egg jelly precipitating activity of the extracts has not been examined in detail. However, it is likely that the three antigens, *b*, *c*, and *d*, of "frozen-thawed extracts" are not related to the egg jelly precipitating activity because the latter activity is removed by high speed centrifugation whereas the *b*, *c*, *d* antigens are not sedimented by such centrifugation (Köhler and Metz, 1959b). With regard to the relationship of the soluble antigens to sperm surface antigens it should be recalled that absorption of anti-sperm serum with "frozen-thawed extracts" lowers the titer but does not completely neutralize the sperm agglutinating action of such antiserum (Köhler and Metz, 1960). This shows that the extracts contain some but not all of the sperm surface antigens. Agar diffusion experiments employing sera absorbed with whole sperm might reveal whether the soluble antigens are surface or subsurface material.

SUMMARY

1. Extracts were prepared from *Arbacia* sperm by several procedures. These were tested for antigenic composition by diffusion against anti-*Arbacia* sperm rabbit serum on Ouchterlony plates and by means of immunoelectrophoresis in agar gel.

2. Extracts prepared by freeze-thawing the sperm followed by low speed centrifugation produced a maximum of four precipitin bands. It is concluded that such extracts contain at least four soluble antigens.

3. Seminal plasma revealed seven arcs in an immunoelectrophoretic experiment, and a maximum of three bands on Ouchterlony plates. Two such bands join bands from "frozen-thawed extracts."

4. Extracts prepared by heating sperm at 100° C. yielded at the most one band. This antigen seemed to be common to several other extracts.

5. Nucleoprotein (Mirsky and Pollister) and histone failed to form precipitin bands.

6. One of the four bands in frozen-thawed extract is associated with material sedimented at 26,000 × *g*.

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SWIMMING SOUNDS AND THE SCHOOLING OF FISHES[1,2,3]

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This paper deals with the swimming sounds of some Bermuda fishes; it describes these sounds and relates them to the behavior of free fishes. E. V. Shishkova (1958a, 1958b) has recently published spectrograms of the sound produced by the movement of schools of scad (*Trachurus trachurus*, Carangidae) in the Black Sea. Such sounds are of interest in their possible significance to the behavior of fishes at sea, and as a reflection of fish behavior. The swimming sounds of fishes offer a possible means of control of fish school movements. The laughter which Hérubel derived (1912, p. 127) from the idea of controlling schools of herring is gradually fading. Sounds engendered by clupeids and the closely related engraulids are hitherto unreported, although a questionable account of herring sounds exists (Murray, 1831), and Maine fishermen describe sound stemming from large herring schools.

"Swimming sound" here conveys that collection of noises created in the water by movements of fish bodies, which characterize the movements of a given species. Such sound may stem from the rubbing together of skeletal parts, from the contraction of swimming muscles, or from water disturbed by various body movements. Shishkova refers to the swimming sound of *T. trachurus* as "hydrodynamic noise," which term she also applies to that noise caused by water flowing along a ship's hull; her data do not permit determining to what extent other events than water flowing over fish bodies contribute to the sound she describes. Since a similar criticism applies to my data, the more general term "swimming sound" seems preferable. Swimming sound is incidental to swimming and includes only those noises characteristically produced during swimming of a given species.

Listening equipment used in the investigation consisted of an AX-58-C Rochelle salt hydrophone and a Woods Hole Suitcase amplifier. Recordings were made during the summer of 1958 on a PT6-BN and a PT6BA2HZ Magnecorder tape recorder at 3¾, 7½ and 15 inches/sec., and analyzed on a Kay Vibralyzer vibration frequency analyzer. Recordings were played back into the water with an Ekotape tape recorder Model 205, a Craftsman C550 amplifier and a QBG transducer. Recordings at sea were made from the motor launch ABUDEFDUF of the Bermuda Biological Station, Mr. Brunell Spurling, Captain.

[1] Contribution No. 1126 from the Woods Hole Oceanographic Institution.

[2] Contribution No. 271 from the Bermuda Biological Station.

[3] The work was performed at the Bermuda Biological Station and at Bowdoin College under Grant NSF-G4403 of the National Science Foundation, and through assistance of the Woods Hole Oceanographic Institution and of the Bowdoin College Faculty Research Fund established by the Class of 1928.

Swimming Sounds of Some Bermuda Fishes

Anchoviella choerostoma (Goode)—Hog-mouth fry. This small engraulid occurred in large and small schools on Bermuda shores during the summer of 1958. The schools were most commonly seen in shallow bays along the north shore of St. George's and Hamilton Islands where they are said to be driven by the commonly observed activity of predators (chiefly carangids and lutianids) feeding on the schools (B. Spurling, personal communication). *Menidia* (Atherinidae) is probably similarly driven on Long Island shores (Butner and Brattstrom, 1960). E. L. Mark (1905) described some Bermuda fishes as gathering schools of smaller fishes to lure prey for the former.

Once established in a bay, a school may remain for several days and even for weeks, gradually reducing in size under peripheral predation and the depredations of fishermen obtaining bait for which the fry are highly valued. A large school darkens the water so that it may be seen over a considerable distance, and the experienced Bermuda boatman can readily distinguish between that discoloration caused by a fish school and that due to rock formation and weed patches.

Observations on the behavior of a large school at sea, estimated to contain several million individuals, of *A. choerostoma* were obtained mainly in Bailey's Bay on August 7, 1958. The launch was repeatedly brought over the school which moved in and out relative to shore during recording. Recordings were obtained when the sea surface was calm and the only extraneous noise stemmed from the system and from snapping shrimp.

No predators were seen feeding during recording; activity around the borders of *A. choerostoma* schools on other occasions consisted of feeding by jacks (*Caranx* spp.) and pompano (*Trachinotus palometa*). Small *Caranx latus* (2 to 6 inches) were frequently netted with *A. choerostoma* and individuals were tolerated as members of small schools of the latter (up to 1000) maintained in the laboratory; the jacks fed on the host schools. *Jenkinsia* and *Sardinella* (Clupeidae) were also taken with *Anchoviella* on occasion.

For recording, the launch drifted over the Bailey's Bay school, engine off; although not obviously so, the behavior of the school was probably modified to some extent by the presence of the boat. During recording, the school was either lying at rest with individual fish turning slowly within a narrow circumference, or the

Table 1

Characteristics of swimming sounds

Species	Size	Sound	Maximum frequency	Frequency of greatest intensity	Time duration
Anchoviella choerostoma	3 inches	Streaming	1.6 kc.	Below .5 kc.	Variable
		Veering of school	2 kc.	Below .8 kc.	.2–.6 sec.
Caranx latus	2–6 inches	Veering of individual	1.7 kc.	Below 1 kc.	.03 sec.
Caranx ruber	1 foot	Veering of individual	1.6 kc.	Below .7 kc.	.05 sec.
Trachinotus palometa	1 foot	Veering of individual	.7 kc.	Below .25 kc.	.06 sec.

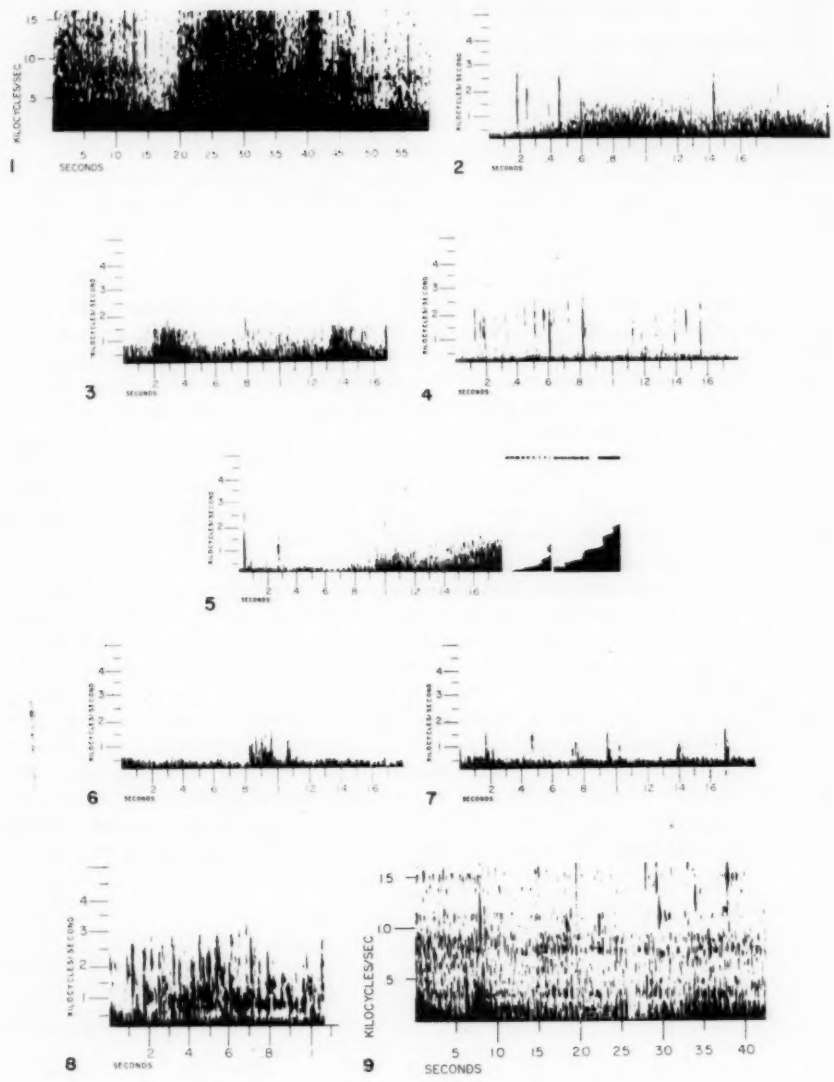

FIGURE 1. Streaming, resting and veering of *Anchoviella choerostoma.*
FIGURE 2. Beginning of a streaming movement of *A. choerostoma.*
FIGURE 3. Two veerings during streaming of *A. choerostoma.*
FIGURE 4. Background and system noise during recording at sea in Bailey's Bay, August 7, 1958.

school had assumed a single direction and the fish were streaming around and by the hydrophone, or the school in the process of streaming suddenly veered in direction with a resultant sharp increase in sound intensity.

Sound could not be recorded from the school at rest; streaming and veering both produced considerable sound (Table I). Sounds similar to these are aptly described by Shishkova (1958a) for *T. trachurus* as being like a pouring or a splash and like a rise and fall in system noise. The greater the degree of veering, the greater the intensity of its noise. The school streamed slowly and rapidly past the hydrophone; sound intensity fluctuated with variations in speed of movement of the school.

Suitcase amplifier and Magnecorder settings varied from 0 + 4–10 and 20–3 db, respectively, to 0 + 14–7 and 30–3 db during recording; at all settings, some veerings of the school overloaded the system.

The entire school covered approximately half an acre; during the observation period, the school broke occasionally into two or three smaller groups. The school and its subdivisions tended to disperse somewhat and gather closely again, so that concentration of fish varied. At times, the school appeared layered, a thinner upper stratum moving in a different direction than the lower bulk of the school. All recordings were done over sandy bottom while the school lay in 2 to 4 fathoms of water; the hydrophone was lowered to mid-depth of the school. When streaming, the school moved past the hydrophone leaving a circular area clear of fish, extending about 6 inches from the casing. With a casing of different color, this interval might be expected to vary (Breder, 1951).

When the school was at rest, it could be stimulated to move by the slightest flexing and stiffening of the observer's knees aboard the 21-foot launch when visual clues were excluded. The casting of a shadow over the school by hand and body movements did not cause this behavior. If the school were at rest when pressure was applied to the deck of the boat by the knee-bend method, it would begin to stream; if it were streaming, it would veer and usually alter the speed of swimming to some extent.

The lower portion (up to 1.5 kc.) of a frequency analysis of sounds stemming from a typical sequence of movements of the *Anchoviella* school is shown in Figure 1. During the 6 seconds of recording illustrated, the school came nearly to rest, began moving again at 2 seconds, veered a few times between 2 and 5 seconds, then quieted again. Abbreviated vertical streaks in the background are due to snapping shrimp; some system noise is indicated below .25 kc.

A similar sequence of events is shown in Figure 2 over a broader frequency scale (5 kc.) and briefer time interval (1.7 seconds). Here the beginning of a streaming movement is shown, again with a few snapping shrimp spectra in the background. Figure 3 shows the spectra of two veerings of the school during a streaming movement. In each of these records, there has been sufficient attenua-

FIGURE 5. Sections through spectrum of recording of *A. chocrostoma* school while at rest and while streaming.

FIGURE 6. Veering of *Caranx latus* school.

FIGURE 7. Feeding and swimming sounds of *Caranx latus*.

FIGURE 8. Veering of *Caranx ruber*.

FIGURE 9. Veering of *Trachinotus palometa*.

tion to bring the schooling movements clearly into the foreground. In Figure 4, the ambient underwater sound of the area (mainly snapping shrimp) and system noise is shown after the *Anchoviella* school had moved inshore from the launch on one occasion.

Figure 5 shows sound intensity spectra (sections) during a recording made from the school at rest and after it had begun to stream. The first section was taken at .6 second of the time scale (school at rest), and the other at 1.3 seconds (school streaming). These sections provide a qualitative indication of the amount of sound stemming from a streaming movement at various frequencies at a point in time. Swimming sound of another *Anchoviella* school of considerably smaller dimensions could not be separated from background noise, due mainly to wave action during a moderate sea state with the equipment employed. In summary, *Anchoviella* swimming sound lies mainly below 2 kc. (Table I). Veering of the school introduces sharp intensity increases of between .2 and .6 second duration.

Attempts failed to record the swimming sound of *Anchoviella* in the laboratory from groups of 500 to 1000 individuals; the sound did not rise above extraneous noise. These experiments were performed in a cement tank, the fish being confined by a wood and plastic screen to an area 8 feet × 2 feet × 1 foot deep. The AX-58-C hydrophone was placed across the tank and fish driven around it. This paper presents evidence that the swimming activity of even small groups of *Anchoviella* is probably important in maintaining the coherence of the school; the swimming sound of a large school is one component of this activity.

A school of blue-fry (*Atherina harringtonensis*, Atherinidae) passing slowly under the launch on August 7 near Bay Island in Bailey's Bay did not respond to the knee-bend method successful in stimulating streaming and veering in *Anchoviella*. The blue-fry school was in the form of a compact ball of perhaps 1000 fish, each turning in its own particular pathway within the school as the latter passed beneath the boat. No swimming sound could be detected.

Caranx latus Agassiz and *C. ruber* (Bloch)—Yellow Jack and Skip Jack. These carangids, common in Bermuda, feed voraciously on *Anchoviella* as well as on other fishes. The *C. latus*, from 2 to 6 inches in length, were captured in small numbers with *Anchoviella* and separately off the dock of the Bermuda Biological Station. Observations are based mainly on aquarium experiments. Two large impounded schools of adult *Caranx ruber* were recorded on the west side of Coney Island on June 26 and August 6, 1958, through the kindness of a commercial fisherman (Mr. Spurling, Senior), and a third school was recorded during underwater listening in Castle Roads on July 11.

Since pharyngeal tooth stridulation may accompany the swimming sound of *Caranx* spp., this habit bears comment. Pharyngeal tooth stridulation of *Caranx hippos* has been described (Burkenroad, 1931; Fish, 1948; Moulton, 1958), and Fish (1948, 1954) describes the stridulating sounds of a number of other carangids. The pharyngeal tooth stridulation is produced more readily by young jacks hand-held gently under water (*C. latus*, *C. hippos*) than by adult jacks so treated (Moulton, 1958, p. 364); it was recorded at Bermuda from adult *C. ruber* held next to the hydroprone in an impounded school, and by *C. crysos* speared off Nonsuch Island on August 17.

Several attempts failed to elicit pharyngeal tooth stridulation from adult pom-

pano (*Trachinotus palometa*, Carangidae) and *Calamus bajanado* (blue-bone porgy, Sparidae), both of which are equipped with formidable pharyngeal teeth. Adult grunts (*Haemulon sciurus*, Pomadasyidae) of some species, on the other hand, stridulate readily when similarly treated.

The present account deals mainly with the thumping sound produced by veering of *Caranx* spp., but pharyngeal tooth stridulation is also produced at times during this maneuver.

Several small groups (from 12 to 20) of young *C. latus* were confined in aquaria 1½ feet × 1¼ feet × 1 foot deep with the hydrophone. Undisturbed, these fish milled about the aquarium equidistant from each other, each on its own pathway. When a hand was flicked at the aquarium or a stick poked into the water, the small group of jacks became at once a tightly coherent school, streaming about the aquarium and veering on repetitive stimuli as had the *Anchoviella* at sea. Streaming did not introduce sound detectable above background; veering did. This movement even of individual fish resulted in distinct thumps similar to those described by Fish (1954) from shocked *C. crysos* (7.75 mm.?). When stemming from a group of *C. latus*, the thumps occurred in volleys (Fig. 6, Table I).

During feeding on bits of meat, the *C. latus* dispersed through the aquarium. The thumps then became scattered as individuals darted for food, and were accompanied by occasional sounds of the stridulation of teeth (Fig. 7).

During the Coney Island recordings, the hydrophone was hung in the midst of schools of *C. ruber* before and during raising of the net. Veering of the fish about the hydrophone caused volleys of thump-like sounds similar to those of *C. latus*, but somewhat deeper in predominant tone (Fig. 8, Table I). Swimming sound engendered by streaming movements was also detected in these recordings and that obtained at Castle Roads.

Trachinotus palometa Regan—Gaff-topsail Pompano. The swimming sound of two pompano approximately 1 foot long was recorded in the cement tank. The animals were not fed during recording. Only thumps coincident with veering occasioned by sudden hand movements in the water were recorded. Sound spectrograms of two flurries of activity are illustrated (Fig. 9, Table I).

Diplodus argenteus (C. and V.)—Bream. Attempts failed to record a swimming sound from 6 of these fish (Sparidae) approximately 8 inches long in a Station aquarium. The only sound detected was made by the fish bumping against the sides and bottom of the tank as a probe was moved gently about the aquarium. These sounds are not clear and sharp like carangid thumps; they are variable and random and are not clearly correlated with describable behavioral phenomena.

Various spear fishermen at Bermuda during 1958, including reliable observers from the Station, reported hearing sounds underwater produced during evasive swimming of grouper (Serranidae) and parrotfish (Scaridae), "any large fish," and hogfish (*Lachnolaimus maximus*, Labridae), as well as grunts of the hamlet or Nassau grouper (*Epinephalus striatus*) and volleys of squirrelfish (*Holocentrus ascensionis*) sound (Moulton, 1958).

The Playback of Underwater Sound to Fish Schools

The playback of underwater sound has been useful in modifying fish behavior (Moulton, 1956a, 1956b; Tavolga, 1956, 1958). The habit of *Caranx* of feeding

on *Anchoviella* led to playing back to the *Anchoviella* school recorded on August 7 recordings of the stridulation sound of *C. latus* and of the swimming sound which accompanies its feeding. The transducer was lowered to mid-school level. The *Anchoviella* cleared the area beneath the launch more quickly during this playback than during recording. Further, during sound playback, the school showed a greater tendency to divide than during recording.

A collateral observation is of interest: When playback began of the stridulation sound at sea, an adult barracuda (*Sphyraena barracuda,* Sphyraenidae) came abruptly to a spot about 8 feet from the suspended transducer and lay quietly facing it for about three minutes. This behavior was unique to all observers, including Mr. Spurling, an experienced Bermuda fisherman and boat captain. (Mr. Spurling's comment: "I've never seen a barracuda act like that before.")

Stridulating sounds and thumps of young *C. latus* played back to about 500 *Anchoviella* in the Station cement tank were accompanied by quickened swimming and milling which gradually subsided. Approximately one-fourth of the fry moved to the opposite end of the tank over and under a horizontally suspended hydrophone which they did not pass spontaneously before sound transmission, and during hand movements in the tank.

Playback to the impounded school of *C. ruber* recorded at Coney Island on August 6, as it milled within the net, of a series of pharyngeal tooth stridulations of *C. latus* recorded in Station aquaria, initially was accompanied by an immediate movement of the school to the far end of the net. After some minutes of playback, the school failed to respond to these sounds and to feeding sounds. These sounds were listened to by surface divers and sounded like the amplified sounds heard during recording.

After the swimming sound of the large school of *Anchoviella* had been recorded at sea on August 7, the recording was played back into an aquarium containing young *C. latus*. The latter showed quickened swimming movements of a non-directional type. It has already been mentioned that small *C. latus* may join *Anchoviella* schools, feeding on the members of the school; they may be attracted to the school by its swimming sound.

Pharyngeal tooth rasps played back to the source species (*C. latus*) in a Station aquarium on August 1 appeared to initiate feeding reaction. The fish became exceedingly active, swimming about furiously, and facing the transducer to nibble at its rubber surface. After several minutes the activity subsided and the fish swam about as usual.

Swimming Movements and Schooling Behavior of Caranx and Anchoviella

The normal behavior of schooling fishes. Caranx, Trachinotus, and *Anchoviella* are truly schooling fishes, and whether at sea or in aquaria, groups of these fishes form cohesive schools. Their behavior bears examination as it relates to factors maintaining the school at sea.

When adult pompano attacked *Anchoviella* schools at sea, the former were in compact school formation, and usually swam directly through the school of fry; feeding of *Caranx crysos* at the periphery of a school was characterized by irregular darting movements and swift swimming of individual jacks.

At rest in aquaria and at sea, the *Anchoviella* school is clearly outlined, although individuals take independent pathways within the school in daytime as at night. When the school is streaming, and when a schooling fish (*Menidia menidia*) of the Woods Hole area is subjected to a tidal current in a live car, these fishes line up parallel to each other and veer together upon appropriate stimulus.

The importance of water currents in mediating behavior of fishes is further underlined by the behavior of small amberjack (2-inch *Seriola* sp., Carangidae) lurking under sargassum weed clumps southeast of Bermuda on August 18. The fish abandoned each clump as the 65-foot *Panulirus* drifted down upon it, and swam directly to another clump, often some yards away but always along the same line of weed and thus within the same convergent zone (Woodcock, 1944, 1950). Due to a combination of factors, significantly wind and Coriolis force, sargassum weed in the open sea tends to arrange itself in parallel rows, the rows being spaced at quite regular intervals under a given set of conditions, and parallel to wind direction (Woodcock, 1944, 1950). Water currents, probably even micro-currents, are of great importance in the open sea in determining the distribution of fishes (see Hasler, 1956); persistent sensory orientation to a particular current or moving body of water may be of considerable importance in maintaining not only the position but also the cohesiveness of fish schools, particularly through hours of darkness (Moulton, 1957a).

Members of a single school vary in their behavior. Schools of 500 to 1000 *Anchoviella* in the cement tank tended to divide at rest into two or three smaller groups; one group was usually markedly larger than the other(s). These groups, treated alike from capture, differed in sensitivity to stimuli; some were easily frightened, others held their position in the face of a variety of stimuli (*e.g.*, a prodding stick or hand).

In a study of variation in reactions to stimuli, a plastic screen confined a single school of about 1000 *Anchoviella* to an 8-foot section of the cement tank during attempts to record swimming sound. The hydrophone was placed across the tank, 3½ feet from the end containing the fish. During transmission of jack stridulation recordings in the area containing the fish, approximately one-fourth of the school moved past the hydrophone. Pushing down on a small board floating over the school (in simulation of the knee-bend method) caused approximately one-half the remaining fish to pass the hydrophone. Then a hand was waved vigorously in the water with the balance of the school; a recalcitrant two dozen fish still remained in the smaller portion of the tank, gathered in a cohesive school.

Young *Caranx latus* tend to school with *Anchoviella;* in an aquarium, a school of 12 of the former formed a tight formation immediately above the back of a 10-inch squirrelfish (*Holocentrus ascensionis*, Holocentridae). They turned with the squirrelfish as it moved slowly about the tank; the latter made no move toward the jacks. Another group of 20 *C. latus* was confined for several hours in an aquarium with an adult spiny lobster (*Panulirus argus*). So long as they were contained together, night and day, the *C. latus* remained at the top of the tank in a tight school. The spiny lobster was removed one night under artificial light, and at once the 20 jacks dispersed over the bottom of the aquarium and assumed a striking striped pattern completely unlike their usual drab coloration.

Experiments on blinded fish. In order to examine factors other than vision

mediating the schooling of fish, adult *Anchoviella* were blinded by bilateral eye removal under anaesthesia (MS222 1:3000, Sandoz Pharmaceuticals) and allowed to recover, or became blinded through eye loss in a syndrome developing within hours of capture in a school held in the cement tank. On successive trials, a blind individual was placed in an aquarium with 12 normal individuals. When the latter were at rest, individuals milling slowly about, the blinded individuals swam through the group and about the aquarium generally until reaching the side of the tank, then took up new headings; they did not orient to the normal fish.

If the normal fish were startled by a hand movement next to the aquarium, they streamed and veered as did large schools at sea; at such times, the blinded individual immediately joined the normal fish in school formation and behaved as did the latter. Similarly, in the cement tank, blinded *Anchoviella* moved with the larger group of normal fish during streaming movements. When the normal fish came to rest again, blinded individuals returned to random movements, while even at rest the normal fish maintained approximately equal intervals between individuals. Isolated blinded fish did not respond to hand movements adjacent to the aquarium.

Unilaterally blinded *Anchoviella* maintained position with the normal fish when the latter were within the field of vision. This latter observation is in agreement with observations obtained on *Menidia* behavior at Woods Hole in 1957, where the experiments were performed in a live car suspended from a raft in Great Harbor; bilaterally blinded fish swam directly through groups of normal fish at rest and oriented independently of normal fish in currents flowing through the live car, while unilaterally blinded specimens maintained position with schools located at least in part on the side of the unoperated eye (Moulton, 1957a).

While movements of the individual members of the fish school are important in maintaining the remarkable integrity of the school, the members of the school are probably sensitive to pressure waves created by the school and probably orient to these waves; the movements of the school as a whole are probably important in maintaining its own integration.

Anchoviella did not disperse through the cement tank after dark as *Jenkinsia* (Breder, 1951) and chub mackerel—*Scomber colias*, Scombridae (Shlaifer, 1942)—do in aquaria. Schools of 500 to 1000 were in tight formation in the center of the cement tank in resting behavior during several examination periods on dark nights when an electric light was turned on, and behavior did not change notably with the light; the basement-located tank was too dark on moonless nights for me to see the fish in it.

C. latus in small numbers confined alone in darkness for several hours did not noticeably change their distribution when a light was turned on. Neither *C. latus* nor *Anchoviella* seems to depend so markedly on light for schooling formation as apparently do *Jenkinsia* and *Scomber*.

A Ghana Fishery Depending on Swimming Sound

A. P. Brown, in an anthropological account of the fishing industry of the Labadi District of the Gold Coast (Irvine, 1947, p. 25), mentioned the three-pronged paddles found in the sea-going canoes of the Coast, without indication as to their

use. Inquiry was made subsequent to a United Nations news release suggesting an application to the fisheries.

The paddle, for possession of one of which I am indebted to Mr. D. A. Hammond of the Fisheries Department, Accra, is in typical form 4 feet 9 inches in length with a handle 1¾ inches in diameter. The foot-long flattened blade terminates in three broad, blunt teeth, comprising 4½ inches of the blade length, each approximately 2 inches in width at the base, the center one tapering slightly more than the other two. Mr. Hammond describes the use of this paddle as follows:

"The use of the paddle as (a) hearing aid in fishing is well-known to our fishermen from Ada to Takoradi. It is particularly used by the herring fishermen. 'Herring' here refers to *Sardinella aurita* and *S. cameroonensis*. It is also used, however, to detect shoals of shad (*Ethmalosa dorsalis*), long-finned herring (*Ilisha melanota*) and cassava fish (*Cynoscion senegalla*). (Identifications from Irvine, 1947).

"Where there are no surface fish visible, the method is to place the broad part of the paddle in the water over the stern of the canoe, place the ear to the top of the handle, and rotate the paddle very slowly. Only skillful and experienced fishermen are appointed to do it. The face of the paddle acts as a sounding board and receives the vibrations which it transmits to the hearer. By rotating the paddle, the fisherman is able to get the direction of movement of the fish and from the intensity of the sound, he can judge how (distant) the fish (are). Those fish which move in large shoals—for example, herring or shad, as well as the large rocks in the sea, make distinct sounds. From the sound, the fisherman can tell whether it is a fish or rock. The sound of the herring is characteristic and never mistaken. This method is particularly successful in waters of 7 to 10 fathoms and between 11 P.M. and 3 A.M. Those paddles which are particularly good are treasured. Some of them are very old." A variety of the Ghana-type paddle is said to be used for a similar purpose in Liberia (Mr. William Watkins, personal communication).

Underwater listening occurs in various parts of the world as an important aspect of commercial fisheries (Cousteau, 1953; Kesteven, 1949; Marshall, 1954; Parry, 1954; Westenberg, 1953). It is likely that in the fishery described by Mr. Hammond, the swimming sound of the clupeids (sciaenids may produce other sounds) is conveyed by the three-pronged paddle to the fisherman's ear.

Discussion

The schooling behavior of teleosts probably depends primarily on vision in the daytime (Shlaifer, 1942; Breder, 1951). Yet schools of *Anchoviella, Clupea harengus* and other schooling fishes maintain their schools at sea through hours of darkness; herring fishermen depend on this fact in "lighting up" herring schools at night (Moulton and Backus, 1955). While it may be that light stemming from luminescent organisms and that of moon and stars on clear nights may afford sufficient stimulus to abet schooling at night, some conditions of darkness at sea undoubtedly preclude vision and require other stimuli as predominant in providing for maintenance of the fish school at night. Certainly other factors than vision enable blinded fish (*Anchoviella, Menidia*) to move with their respective schools.

The possible role of water currents in maintaining fish schools at sea has been briefly discussed.

Significant factors in low frequency sensitivity of teleosts are the lateral line and isolated cutaneous receptors (von Frisch, 1938; Griffin, 1950; Lowenstein, 1957). Tracy (1920a, 1920b) and others have suggested that the connection between air bladder and inner ear of clupeids (engraulids possess a similar connection—Berg, 1947) may be more important as a pressure control device related to gas physiology of the air bladder than to hearing facility. While it is not unlikely that the paired passages may serve in both capacities, the remarkable sensitivity of *Anchoviella*, as well as of some clupeids (Moulton and Backus, 1955; Moulton, 1956a), to pressure waves in the water is suggestive of special adaptations to reception of pressure changes.

Anchoviella are sensitive to very slight pressure waves in the water; the swimming sounds described fall within the frequency range of sensitivity of all fishes studied in this connection. It is now simply conjectural, although some supporting evidence has been presented, to suggest that the swimming sounds of various carangids and of *Anchoviella* furnish a mechanism in addition to water currents and visual sensitivity by which schools of fish at sea are maintained under a variety of circumstances. Either these sounds or the body movements from which they stem must be a primary factor.

The swimming sounds described are to a degree species-specific. Shlaifer (1942) observed that chub mackerel, pairs of which will school together, will not school with abnormally moving fish of the same species. Normal *Menidia* and *Anchoviella* do not arrange themselves in schooling formation with blinded individuals; the latter will school with the former. It is suggested that the fish school at sea is maintained by a number of factors, given the proper amounts of food and minimum predation: sensitivity to particular water currents and masses, vision, and the behavior of the school as a whole which acts an an acoustical core and as a wave pressure source to which individuals of the schooling species orient. It is suggested that the swimming sound is not simply a mechanical or accidental sound, but that it possesses biological significance. It is demonstrated that source species and other species may react to amplified swimming sound.

Another factor influencing the maintenance of the fish school is reflected in differential sensitivity of *Anchoviella* to increasingly dispersive stimuli. After a series of such stimuli, a core of recalcitrant individuals remains in the test area in an experimental tank, and may furnish the nucleus around which more sensitive members of the school fluctuate at sea; streaming and veering may orient around a group of relatively insensitive individuals forming an "anchor" to the school.

It is clear that the swimming sound of different species of schooling fishes (*Anchoviella, Caranx, Trachinotus*) varies in its characteristics with species as well as with size of the fishes concerned. Large schools of fishes as small as *Anchoviella* (about 3 inches) and as large as *Caranx ruber* (about 1 foot) introduce considerable sound into the water. This may in future be utilized in experiments on schooling fishes in waters less clear than those studied, to test influence of various stimuli on schooling fishes and to explore sensitivity to these stimuli. Although Shishkova (1958a) abandoned the hope of stemming acoustically the flow of anchovies from the Russian Sea of Azov into the Black Sea, and of masking ship

noise with swimming sound, other possible uses of swimming sound exist; a likelihood is present that fishermen can control acoustically to some extent the movements of clupeid and engraulid schools.

The characteristics of these sounds are more like those of sounds produced under circumstances related to defensive behavior of fishes than of sounds produced under other circumstances (Moulton, 1957b). The sound attendant on veering may have some protective value. If this were true, large schools of herring and anchovies would be more resistant to predation by other fishes than would small schools. The joining of *Anchoviella* schools by young *C. latus* may be primarily protective for the latter; their feeding on the host school would simply indicate poor "guestmanship."

The swimming sounds described, as well as sound stemming from the movement of any large fish in the sea, are probably due to a number of factors; hydrodynamic noise, to which Shishkova relates all of the sound, skeletal movements, and contraction of axial musculature during strong swimming movements against a gas-filled, resonating air bladder. None of the sounds described are continuous even during steady streaming movements, indicating that they stem from the bodies of individual fishes during muscle contraction, rather than from the hydrodynamic effects of continuous water flow around fish bodies.

All of the swimming sounds studied lie below 2 kc., and thus are of lower frequencies than many sounds created by fishes by organs apparently specialized for sound production (Moulton, 1958). These results differ from those of Shishkova (1958b) who found components of *T. trachurus* swimming sound at 16 kc. (It seems likely that her fish were chewing or stridulating during recording.) The frequency span of greatest intensity of swimming sound, as indicated by darkening of vibragrams, is lower for large fish than for small (Table I).

After submission of the manuscript of this paper for publication, the author's attention was called to a recent extensive study of social groupings in fishes by Breder (1959). Of special pertinence to the present work is Breder's caution that the responses of members of a fish school to light and darkness will vary with individuals, and that light exposure which fish have received earlier will be of great importance.

The author is grateful to Dr. Richard H. Backus for constructive criticism of the manuscript of this paper.

Summary

1. The swimming sounds of four schooling species of Bermuda fishes are described. The sounds stem from streaming and veering of schools at sea.

2. Responses of schooling species to playback of sounds stemming from schools are described. Exploitation of these responses in experimental and commercial fishing is suggested.

3. On the basis of observation at sea and of laboratory experiments, mechanisms useful in maintaining formation of schools at sea are discussed. These include vision, water currents and pressure waves initiated by the movements of fish bodies and the sounds they engender.

4. An African acoustical fishery, probably relying on the swimming sounds of fishes, is described.

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BINUCLEATE AND TRINUCLEATE OOCYTES IN POST-OVULATION OVARIES OF RANA PIPIENS[1]

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The senior author had long been interested in the means by which diploidy can be produced in parthenogenetically developed frogs and larvae (Parmenter, 1920, 1925, 1933, 1940, 1952). He demonstrated (1952) the existence of the diploid chromosome number in three mature virgin eggs and considered this to be one source of diploidy with a known history of no delay in cleavage. He had found one instance (1940) of a diploid many-celled embryo in which, by direct observation, he knew that both polar bodies were given off and that it developed with no delayed cleavage. The discovery of a small number of binucleate ovarian eggs suggested another important possible source of such diploid parthenogenetic individuals.

In the course of undergraduate research in which nuclei were dissected from ovarian oocytes of *Rana pipiens*, several eggs were found which possessed two completely separate nuclei, and it was established that the binucleate condition was not an artifact of manipulation. The eggs used were in those stages where the nuclei had reached their fullest growth (stages 4, 5, and 6 of Duryee, 1950). Subsequently, a method was devised for scanning in ovarian tissue a large number of transparent oocytes in such early stages (stages 1 to 3 of Duryee, 1950) that the yolk would not interfere with direct observation of the cells *in situ*. It was hoped eventually that the chromosome content could be determined.

Material and Methods

Technique. The sac-like ovaries of female frogs in the post-ovulated condition were opened along their edges in Ringer solution by means of fine-pointed jeweler's forceps. The two halves, or sometimes individual pieces only, were floated onto a slide. They were scanned under low power (16 mm. objective) in the living condition, or were fixed in Bouin's fluid (which served as a stain), dehydrated, and mounted in damar. In all cases the slides were systematically surveyed and all eggs counted in which there were visible nuclei. Some cells were examined and measured first unfixed and then fixed.

[1] Mrs. Parmenter acknowledges with sincere appreciation the encouragement and helpful advice given her in the writing of this paper by Professor D. H. Wenrich; also the kind suggestions of Professors D. R. Goddard, W. R. Duryee, Gerhard Fankhauser, and others.

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[2] All of the data herewith presented were collected and organized by Dr. Parmenter, assisted by Mr. Derezin. Unfortunately Dr. Parmenter died before the writing of the paper, which subsequently was undertaken by his wife.

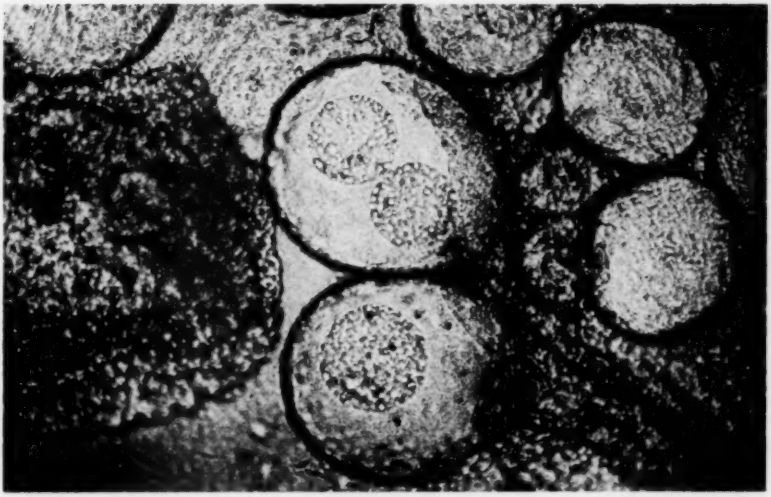

FIGURE 1. Binucleate #2 photographed *in situ* in unfixed ovarian tissue. Adjacent is the mononucleate used as a control for comparative measurements. Included also are younger oocytes and an older yolk-filled one. 430 ×

Measurements. Three different methods of measurement of egg and nucleus diameters were employed, depending on the exigencies of the moment.

Fixed eggs: When an egg with two nuclei was found, it was measured with an ocular micrometer in at least three or four diameters to correct for any variation from the spherical condition. For comparison as a "control" a single-nucleated cell of approximately the same size was located as close in the field as possible and its diameter measured. Similarly the nuclear diameters of both eggs were determined. From these data the volumes of the eggs and of their nuclei were calculated, assuming them to be spherical.

Fresh unfixed material was treated in one of two quite different ways: Usually the living binucleate was photographed *in situ*, together with a nearby mononucleate. In two cases both were fortunately in the same field. (For one, see Figure 1.) Without changing the setting of the microscope or camera, a stage micrometer was also photographed. The films were processed together and enlarged to the same magnification. Measurements were made by means of these photographs. Occasionally, when a camera was not available, camera lucida drawings were carefully made of the living binucleate, its control, and the stage micrometer, and measurements obtained from these drawings.

Extreme care was taken with all calibrations so that the measurements as finally presented in the tables are comparable with each other.

OBSERVATIONS

Fifty-six binucleate and two trinucleate eggs were found among the 249,616 small transparent primary oocytes observed. This total represented all the eggs

with visible nuclei found in 25 frogs. Thus the binucleates constituted a percentage of 0.022% or a ratio of 1 to 4,457 ova.

The percentages of multinucleates in the frogs deviated significantly from one another. Fifteen of the 25 females possessed no multinucleated eggs at all. Of the 10 with multinucleated oocytes, 8 contained very few each, only one to four. However, in the ovaries of two individuals a large number of ova with two nuclei were found, namely 14 or 0.093% in one (#44), and 24 or 0.175% in the other (#39). This indicates that certain females are more prone to produce the multinucleate condition than others where it is determined by chance. This situation, while certainly of physiological significance, is not unusual. The literature abounds with similar cases. Indeed, Parmenter's (1952) rare diploid metaphases were found in three virgin eggs, all of which came from the same female, whereas none appeared in eggs from 11 other frogs. He discussed the literature in some detail (pp. 253–254).

There was considerable variation in the number of eggs found in each frog. The range was from 5,426 to 16,066. There did not appear to be any relationship between the abundance of ova in a female and her size as indicated by the length, measured from nose tip to anus.

Three of the 25 frogs contained ovaries with considerably more eggs than the others, 13,687–16,066. The two most productive of multinucleates were among these, but a frog with 13,717 eggs possessed only one binucleate. Moreover, the first three eggs which were found with two nuclei were in an individual with small ovaries containing a total of only 5,632 eggs.

One could not predict, therefore, from external conditions such as the size of the animal, or abundance of eggs, whether multinucleate oocytes were likely to be present.

The sizes of those oocytes containing either two or three nuclei varied in diameter from very small, 0.099 mm., to 0.350 mm. (Table I). Nine ova were smaller than 0.200 mm., *i.e.*, in "stages 1 or 2" (Duryee, 1950). The rest, including the two trinucleates, were all in "stage 3." The majority of the oocytes (20) were found to measure between 0.200 and 0.300 mm. Even in one female the multinucleated eggs varied markedly in size. In female #39 which produced the 24 binucleates the diameters of the oocytes ranged from 0.163 to 0.323 mm. with most from 0.200 to 0.300 mm. Also in frog #44 (14 bi- and 1 trinucleate) the variation included the tiniest egg of all (0.099 mm.) and extended to 0.292 mm.

It is recognized that within an ovary a condition of egg growth is in progress with the various stages of growth distributed indiscriminately throughout the structure. A suggestion of this is seen in Figure 1 where one can observe a yolk-filled egg of a later stage adjacent to the mono- and binucleates which were compared, and nearby much smaller eggs. It is thus easy to see how the oocytes to be compared were chosen. The senior author was much concerned with the possibility that, due to the extended growth period of primary oocytes, the cells compared, although of the same size, might not have been growing for the same length of time. This difference in the age of the oocytes would not affect the validity of the observations, merely the conjectures as to interpretation.

In an attempt to find a clue to the chromosome content of each nucleus, the nuclear volumes of the bi- or trinucleated cells were determined and compared with that of the nucleus of the normal mononucleate which would serve as a "con-

TABLE I

Occurrence of binucleate and trinucleate oocytes in frogs (Rana pipiens)

A. Binucleate oocytes

Binucleate oocyte number	Frog number	Nuclear volumes ($mm.^3 \times 10^{-4}$)				Cell diameters (mm.)	
		Binucleates			Mononucleate control	Binucleate	Mononucleate control
		Nucleus a	Nucleus b	Sum of a and b			
Binucleates with the volume of both nuclei approximately equal							
1	36	4.94	4.94	9.88	12.3	0.350	0.334
2	36	5.24	5.24	10.5	10.2	0.275	0.260
3	36	6.83	6.83	13.7	10.8	0.303	0.291
8	39	14.2	13.0	27.2	36.0	0.339	0.343
12	39	7.05	6.28	13.3	17.7	0.290	0.290
13	39	5.11	5.72	10.8	7.59	0.215	0.220
14	39	10.4	8.64	19.0	15.3	0.289	0.266
15	39	7.27	6.59	13.9	11.9	0.263	0.265
18	39	2.35	2.04	4.39	3.79	0.162	0.161
19	39	7.16	7.43	14.6	14.0	0.272	0.271
21	39	3.97	4.18	8.15	6.74	0.209	0.208
23	39	5.89	5.89	14.2	15.7	0.280	0.256
24	39	6.59	7.59	14.2	11.5	0.239	0.234
27	39	5.89	5.75	11.6	11.5	0.248	0.276
28	39	10.0	12.5	22.5	23.5	0.323	0.321
29	42	3.79	4.08	7.87	6.09	0.191	0.190
31	44	5.59	5.36	11.0	7.59	0.215	0.225
35	44	1.75	1.65	3.40	3.52	0.137	0.137
36	44	9.14	8.04	17.2	13.1	0.258	0.247
37	44	0.662	0.593	1.26	1.75	0.099	0.102
38	44	5.02	5.46	10.5	21.7	0.292	0.305
39	44	2.86	3.17	6.03	7.00	0.193	0.199
41	44	3.62	4.49	8.11	11.9	0.256	0.251
44	44	3.52	3.52	7.04	5.11	0.183	0.177
45	44	1.03	0.98	2.01	1.81	0.114	0.113
48	48	3.42	4.49	7.91	9.32	0.207	0.215
49	48	7.01	6.89	13.9	15.8	0.245	0.245
51	51	6.09	5.02	11.1	9.07	0.229	0.223
57	59	0.95	0.95	1.90	2.35	0.119	0.120
Binucleates with markedly unequal volumes							
30	44	1.19	0.39	1.58	1.25	0.113	0.112
43	44	8.04	4.90	12.9	8.04	0.237	0.237

B. Trinucleate oocytes

Trinucleate oocyte number	Frog number	Nuclear volumes ($mm^3 \times 10^{-4}$)					Cell diameters (mm.)	
		Trinucleates				Mononucleate control	Trinucleate	Mononucleate control
		Nucleus a	Nucleus b	Nucleus c	Sum of a, b, and c			
1	44	5.96	6.09	6.34	18.4	15.1	0.271	0.266
2	51	3.01	4.37	4.26	11.6	9.32	0.212	0.214

trol." The sum of the volumes of the twin nuclei was approximately equal to that of the single one chosen for comparison in 29 measurable cells, and markedly unequal in 2 (Table IA). Four others possessed what appeared to be unequal nuclei, but due to misshapen cells or nuclei, or visible shrinkage in one or two cases, meaningful measurements seemed impossible. These were omitted from the table, as were three ova with two nuclei of apparently the same size where the conditions were such that the measurements were questionable. The other twenty eggs, although clearly binucleate, could not properly be measured.

In none of the binucleates did the volume of either of the two nuclei approach that of the selected "control," except in the case of egg #43. It will be seen from Table I that the larger nucleus had exactly the same volume as that of the mononucleate control, and the smaller was somewhat more than half that volume. One other difference between the two nuclei of egg #43 besides size should be mentioned. There is a conspicuous dissimilarity in the granular appearance. The smaller one resembles the usual binucleate in the peripheral arrangement of the large chromatic granules (nucleoli), whereas in the larger nucleus these bodies tend to be smaller and distributed more uniformly throughout the nucleoplasm.

The total volume of the twin nuclei that were of the same size approximately equalled that of the single nucleus of the cell chosen for comparison (Table IA). There was one exception also to this statement in the case of egg #38. In this interesting cell the sum of the volumes of the two nuclei was only about one-half the volume of the nucleus of the mononucleate. Measurements of additional control cells confirmed this relationship.

While searching for the cells with two nuclei, unexpectedly the two trinucleates were found. These conformed to the general pattern for binucleates in that the diameters of the cells were in the same size range, and the sum of the volumes of the three nuclei approximated that of a "control" mononucleate. Both were located in ovaries containing cells with two nuclei also. Trinucleate #1 was found in the same lobe as binucleate #43 which possessed the large nucleus equal in size to that of the control. The three nuclei of trinucleate #1 (Table IB) were of about the same size. Since in both cases the three nuclei were at different levels within the cell a photograph was not feasible. In trinucleate #2 two of the nuclei were larger in size than the other, and equal in volume to each other. Interestingly, a similar difference in granular appearance existed between the two large nuclei and the smallest one as was described for binucleate #43. The small nucleus had fewer and larger chromatic granules whereas the two larger nuclei possessed smaller and more numerous ones.

Discussion

The following ideas were found among Doctor Parmenter's notes. No further discussion will be attempted.

The unequal-sized binucleates and the trinucleates suggest that a possible origin may have been from separated groups of chromosome vesicles. But such an origin does not seem probable for the large proportion of binucleated oocytes, the nuclei of which were of equal volume. More likely this condition arose by a nuclear division followed by a failure of cytosomic division. These binucleated cells may have been produced during the last oogonial division.

Should further information demonstrate that in some cases each of the two equal-sized nuclei contains the full set of 13 tetrads, both the normal behavior and the possible failure of one of these nuclei to give off its polar body in either meiotic division would produce various chromosome numbers in mature eggs and in embryos resulting either from parthenogenetic stimulation or from fertilization.

Literature

Information in the literature concerning multinucleate oocytes is sparse. Indeed Humphries (1956) in discussing the origin of spontaneous polyploidy in *Triturus viridescens* hesitated to "assume" (p. 120) the existence of a binucleate oocyte as a source of diploidy in such embryos. He preferred the explanation of (p. 120) "direct pathways actually seen to exist," one of which he describes in his paper on the effects of heat shock on the first meiotic division. We have now shown that binucleate primary oocytes actually do exist, at least in *Rana pipiens*. Beatty (1957) who reviewed the literature on polynuclear ovarian eggs, states that such are (p. 81) "rare but widely spread." He mentions twelve species of mammals (including man), reptiles, birds, insects, but no amphibians. Sentein (1958) was able to produce multinucleated eggs of *Triturus* and *Pleurodeles* by treatment with phenol. He saw cytoplasmic division inhibited. This treatment constitutes, of course, an unnatural source of the polynucleate condition.

In embryonic tissue Parmenter (1937, 1940) reported multinucleated erythrocytes in parthenogenetic frog larvae, also five epithelial cells each with two nuclei plus one with three. Moore (1957) has presented evidence that chromosomal vesicles constitute a basis for the origin of what she refers to as "double nuclei" (p. 209) in early embryos of diploid frog hybrids. She reviewed the literature extensively. Of interest here is her discussion of the distribution of peripheral coarse vs. diffuse fine chromatin in some of the cell nuclei of her material. She states that similar conditions were also found by King and Briggs, by Brachet, and by others. She wonders if some of the (p. 222) "so-called nuclear anomalies" found in hybrids are not really of (p. 222) "normal occurrence in the development of amphibian eggs."

The occurrence of the bi- and multinucleate condition *normally* in liver tissue of mammals including human is well known and has been reviewed recently by Inamdar (1958). By microspectrophotometric measurements of DNA in resting nuclei of mouse liver, he was able to confirm the conclusions of Beams and King (1942) and others that the origin of the binucleates is best explained by division of the nucleus without division of the cell.

No discussion will be undertaken on the often-noted polynucleate condition in tissue cultures, or in pathological material.

Summary

1. Fifty-six binucleated and two trinucleated cells were found among 249,616 young transparent primary oocytes in post-ovulation ovaries of 25 females of *Rana pipiens* (0.022%).

2. Multinucleated oocytes were absent in 15 females, present in 10. Eight of these 10 produced only one to four binucleates each; one female was the source

of 1 tri- and 14 binucleates; and another gave the remarkable number of 24 (0.175%), all binucleates.

3. The multiple nucleated condition did not seem to be correlated in any way with the size of the female, the abundance of her eggs, nor the size of the egg.

4. In none of the binucleates did the volume of either of the two nuclei approach that of the nucleus of a mononucleate of the same size. The one exception was egg #43 where one nucleus did have exactly the volume of the control, the other about half that.

In 29 of the 31 measurable binucleates the two nuclei were of approximately the same size and the sum of the two volumes equalled that of the mononucleate, except in one case where it was half.

The origin of binucleate oocytes remains uncertain; it may be connected with a final division of an oogonial nucleus that was not followed by cell division. In this case the two nuclei would both be diploid.

5. The two trinucleates conformed in general to the same pattern as the binucleates as to their distribution, size of oocyte, and the volumes of their nuclei. The sum of the three nuclear volumes approximated that of the mononucleate.

6. In two cases where the nuclei were markedly unequal in size, there was a definite difference in the appearance of their chromatic granules. These bodies were more abundant and finer in the larger nuclei, peripheral, larger and more distinct in the smaller nucleus.

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COLD DEATH IN THE GUPPY [1]

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There are numerous reports describing the death of vast numbers of fish subsequent to cold waves, often where the water temperature remained well above 0° C. (Wilcox, 1887; Verrill, 1901; Vatova, 1929; Storey and Gudger, 1936; Storey, 1937; Miller, 1940; Gunter, 1941; Gunter, 1947; Mosevich, 1944).

Maurel and Lagriffe (1899) divided the changes induced by placing fish into colder and colder water into five stages. Stage five consisted of convulsions, loss of equilibrium, complete paralysis, and a state of apparent death; the fish would revive only if warmed within a few minutes. If not warmed, they would go on to die without showing any visible signs of life. Doudoroff (1942) called this "primary chill coma."

At less extreme temperatures a different sequence of events occurred. The fish would recover from the initial shock, resume respiration, if it had stopped, and take on a relatively normal appearance. If kept at such a temperature for some hours or days, the fish would show increasing distress and finally cease to respond to stimuli and to respire. This characteristic response to less extreme temperatures, Doudoroff (1942) called "secondary chill coma." In "primary chill coma" the fish die without a return of vital signs, unless rewarmed; in "secondary chill coma," vital signs, if they disappear, return for a time and the fish then gradually die unless rewarmed.

Virtually nothing is known of the mechanism of death due to "primary chill coma." Brett (1952) experimented with cold tolerance in young Pacific salmon and concluded that death occurring in the first hour of a cold exposure was probably due to a disturbance of the central nervous system. It has been shown that with decreased acclimation temperature a lower temperature is required to induce "primary chill coma" (Samochvalova, 1938) and the fish can withstand longer exposures to a lethal cold temperature (Fry, Brett and Clawson, 1942; Doudoroff, 1942; Brett, 1956).

Only Doudoroff (1945) has proposed a mechanism of death due to "secondary chill coma." He showed that the death of *Fundulus*, a salt-water fish, at slowly lethal temperatures in sea water was preceded by osmotic dehydration of tissues, and was delayed in diluted sea water. He concluded that osmoregulative failure was one of the causes of slow death in cold sea water.

The characteristics of cold tolerance in the guppy, *Lebistes reticulatus*, have never been thoroughly explored. The guppy is an ideal subject for inquiry into

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the effects of cold on fish. It is hardy, inexpensive, and easily procured. More than 4500 guppies were used in the past three and one-half years to secure the information presented in this paper.

This paper will propose for the first time a possible mechanism of death due to "primary chill coma" in fish.

Methods and Material

Approximately 1000 guppies were obtained from aquatic plant tubs in the greenhouses of the University of Pennsylvania, Department of Botany. The remainder were bought from Florida fish farms. The fish were acclimated for two weeks to five-, ten-, or fifteen-gallon stock aquaria, with up to 40 fish per gallon of water. The fish were fed various commercial brands of dry fish food and Dash dog food once daily.

Randomly chosen fish were taken from stock aquaria by means of a soft net and quickly placed into the test water which was maintained at uniform temperature (0.1° C.) by gentle shaking of the container unless otherwise stated, by the gradual addition of ice to a constant temperature bath into which the test container

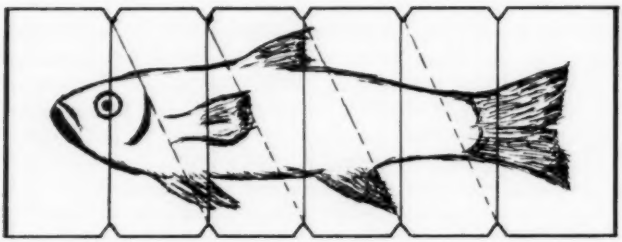

Figure 1. Comatose fish secured to a notched glass slide with a soft cotton thread.

was immersed. Temperatures were read from standardized mercury thermometers graduated to 0.1° C. or 0.05° C. At the completion of a test exposure or a series of stepwise test exposures the fish were quickly removed and placed in water from the stock tank.

Once a fish regained normal respiration and was able to swim normally after a test exposure, it almost invariably lived. Those fish alive 24 hours after test exposures were considered as having survived the insult. Deaths after this 24-hour period were few, always less than 5%, and seemed to depend mostly on the general condition of the fish prior to the cold exposure.

In the experiments in which fish were subjected to cold in oxygen-depleted water or oxygen-enriched water, the water was vigorously bubbled and equilibrated with 100% nitrogen or 100% oxygen for one hour immediately prior to its use. In approximately one-half of these experiments a sample of test water was siphoned directly into a sampling jar at the midpoint of the cold exposure and immediately subjected to a Winkler oxygen determination. In all the experiments where a Winkler oxygen determination was performed, distilled water was used in the

test container, rather than stock tank water, so as to minimize the amount of organic material which might interfere with the accuracy of the oxygen determination. The substitution of distilled water for stock tank water did not alter the results. In those experiments where an oxygen determination was not performed, the oxygen content was estimated and rounded off to the nearest 5 mg./L.

Some of the data to be presented are observations on individual fish of the relationship of respiration to the presence of a functioning circulation. Gill and mouth respiratory movements were easily seen with the naked eye. The criterion for the presence of a functioning circulation was the presence of blood flow in the arteries and veins at the base of the fish's tail. Thirty-five or 100 diameters magnification of these vessels rendered blood flow clearly visible. A fish after being paralyzed in the cold test water was quickly placed on a notched glass slide and gently secured with soft cotton thread as diagrammed in Figure 1. This necessitated removing the fish from the test water for only a few seconds. The slide was replaced into the test container on a microscope stage and the tail vessels brought into focus. The time intervals required for respiration and circulation, respectively, to stop after chilling and to start after warming, were measured by means of a stop-watch. The microscope and other equipment were set up in a constant temperature cold room set at the temperature of the test water. The temperature of the test water remained constant to within 0.2° C. during each experiment.

All data were analyzed statistically by a special method of computing chi square in an Rx2 table (Snedecor, 1956, p. 227).

Results

The guppy's normal temperature range, according to Innes (1955), is 16° C. to 40° C.; Gibson (1954), however limited the upper temperature to 32° C. When a guppy was exposed suddenly to water below 10° C., it exhibited the characteristics of "primary chill coma." Often after a few minutes at the low temperature, the fish's chromatophores expanded, causing the fish's color to darken. Exposures to cold temperatures above 10° C. resulted in "secondary chill coma."

Upon being replaced in a stock tank after "primary chill coma," the fish would usually begin to respire within eight minutes and almost invariably within twenty minutes, if they were to survive. Occasionally a fish would revive, show respiratory movements for several minutes to a few hours and then die. In "secondary chill coma," if a fish had gradually ceased to respire during the cold exposure, it usually did not revive when replaced in stock water of normal temperature, but if it was still respiring when taken from the cold, it usually survived.

I. Primary Chill Coma

A. Study of a single population

Approximately 700 guppies of all sizes were taken from a large aquatic plant tub in a University of Pennsylvania Botany Department greenhouse and acclimated to 23° C. ± 1° C. for 10 days. Batches of 10 adults, 5 males (80–130 mg.) and 5 females (100–900 mg.), and batches of 10 young guppies (6–18 mg.) were randomly picked and subjected to specific cold exposures. The rest of the fish were then acclimated to 30° C. ± 1° C. for 10 days; batches

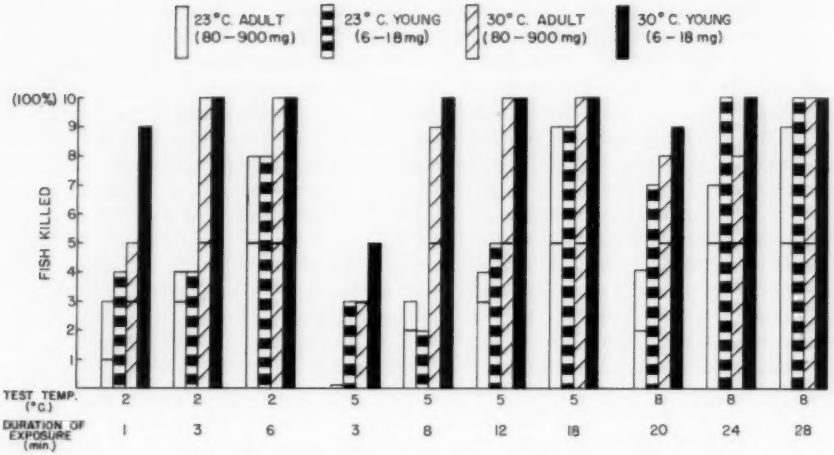
23°C. ADULT (80–900mg)
23° C. YOUNG (6–18mg)
30° C. ADULT (80–900 mg)
30° C. YOUNG (6–18 mg)
(100%) 10
FISH KILLED
TEST. TEMP. (°C.)
DURATION OF EXPOSURE (min.)
2 2 2 5 5 5 5 8 8 8
1 3 6 3 8 12 18 20 24 28

FIGURE 2. Death due to "primary chill coma" in the guppy. The black bar separates the females above from the males below.

of these adults and young were then subjected to the same cold exposures as those fish acclimated to 23° C. The results are summarized in Figures 2 and 5; Figure 2 shows the results of exposures to temperatures causing primary chill coma, and Figure 5 the results of exposures to temperatures causing secondary chill coma.

1. Effect of the temperature, duration of cold exposure, sex, size, and acclimation temperature on mortality

Combining all the data in Figure 2 it is seen that: (1) the lower the temperature, the more rapidly lethal effects occurred; (2) each increase in

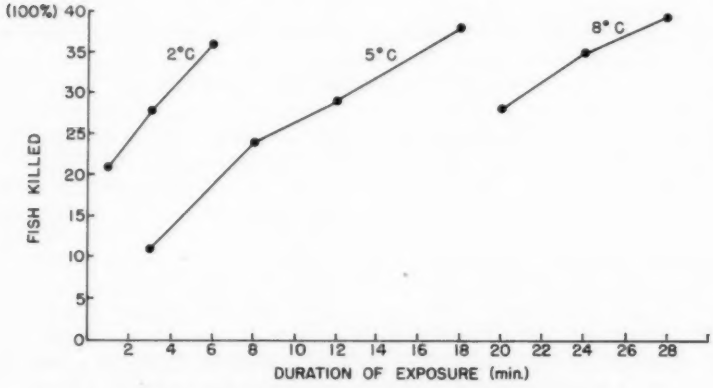

FIGURE 3. Total number of fish killed by each cold exposure at 2° C. ($p < 0.005$), 5° C. ($p << 0.001$), and 8° C. ($p < 0.005$).

the duration of exposure to a specific cold temperature was associated with an increase in the total number killed (Fig. 3); (3) males are less cold-tolerant than females ($p < 0.005$); (4) young are less cold-tolerant than adults ($p = 0.005$); (5) in both young ($p < 0.001$) and adults ($p < 0.001$) tolerance to "primary chill coma" is inversely related to acclimation temperature.

2. Effect of acclimation temperature on tolerance to anoxia at normal temperatures

When placed in 21° C. oxygen-depleted water (less than 0.25 mg. O_2/L.) for 15 minutes, none out of 20 fish acclimated to 23° C. succumbed, yet 10 out of 20 of fish acclimated to 30° C. died ($p = 0.001$). Tolerance to oxygen lack is thus inversely proportional to acclimation temperature.

Sumner and Doudoroff (1938), using boiled sea water and 0.001 molar potassium cyanide in sea water, likewise demonstrated that in the gobie, *Gillichthys mirabilis* Cooper, tolerance to anoxia was inversely related to acclimation temperature.

B. Lethal roles of the rapidness and repetition of chilling

1. Rapid versus slow chilling

In experiments where the water temperature was gradually reduced from the acclimation temperature to 2° C. in 15 minutes or to 8° C. in 7 minutes, 13 out of 16 fish died, whereas with rapid chilling to the same temperatures 13 out of 16 fish also died.

2. Lethal role of repetitive chilling

A batch of 20 adult guppies, 10 males and 10 females, acclimated to 23° C. was placed at 5° C. for one minute and subsequently at 23° C. for

TABLE I

Relationship of circulatory and respiratory functions in death due to "primary chill coma" in the guppy. Fish were acclimated to 29° C. for ten days

Fish No.		When chilled		When placed at 25° C.		Resp. started at	Condition 24 hrs. later
		Resp. stopped at	Circ. stopped at	Circ. started at	Circ. stopped at		
#1	5° C. for 3 min. (¼ LD50)	less than 10 sec.	30 to 80 sec.				Alive
#2	5° C. for 3 min. (¼ LD50)	less than 10 sec.	60 sec.				Alive
#3	5° C. for 5 min. (1 LD50)	3.6 sec.		15 sec.		75 sec.	Alive
#4	5° C. for 5 min. (1 LD50)	4.7 sec.		10 sec.		50 sec.	Alive
#5	2° C. for 3 min. (3 LD50)	2.3 sec.		15 sec.	2 to 11 min.	—	Dead
#6	2° C. for 10 min. (10 LD50)	3.5 sec.		210 sec.	7 to 10 min.	—	Dead
#7	2° C. for 65 min. (65 LD50)	2.6 sec.		—		—	Dead
#8	2° C. for 75 min. (75 LD50)	4.0 sec.		—		—	Dead

one minute. These exposures were repeated six times in succession. Each time the fish were chilled they lost all respiratory and swimming movement; but when they were placed at 23° C., most of them regained respiratory movements. None of these fish died.

C. Relationship of respiratory movements to the presence of a functioning circulation (Table I).

Careful individual observations were made on 30 fish subjected to 2° C., 5° C., or 8° C. Twenty fish received an LD50 exposure, 8 received 3 to 12 times the LD50 exposure, and 2 received over 60 times the LD50 exposure. In each of these 30 fish a complete cessation of all respiratory and swimming movements occurred within 10 seconds. The results were consistent; the data from 8 typical fish of this group are presented in Table I. The circulation, as judged by blood flow in the tail vessels of two fish (Fish #1 and #2, Table I), ceased approximately one minute after the fish were placed at 5° C.

If the heart beat returns it does so before there are any respiratory movements, as described by Britton (1924); in the present experiments if the circulation returned it did so before the respiratory movements, and the respiratory movements never returned unless a functioning circulation was already present.

Fish #3 and #4 (Table I) were subjected to an LD50 cold exposure and only transient respiratory and circulatory depression resulted. In all of the 20 fish subjected to an LD50 cold exposure the circulation returned. Respiration returned in 13 out of the 20 and they survived; in 4 others out of the 20, respiration returned for a brief period, subsequently stopped and the fish died; in the 3 others remaining out of the 20, respiration did not return and they died.

Fish #5 and #6 (Table I) were exposed to cold long enough to permit permanent respiratory depression but only transient circulatory depression. This resulted in the 8 fish exposed to 3 to 12 times the LD50 cold exposure as well as 3 of the 20 fish which received approximately an LD50 cold exposure.

Only 2 of the 30 fish (#7 and #8, Table I) received cold exposures of greater than 12 times the LD50 exposure. Neither the circulation nor the respiration returned in either fish and they of course did not survive.

D. Effect of increasing the tonicity of the chilling water

Fish were chilled at 5° C. for 8 minutes in distilled water, 0.32 molar sodium chloride (F.P. − 1.2° C.), or 0.65 molar glucose (F.P. − 1.2° C.) (the freezing point of teleost body fluid is between − 0.5° C. and − 0.9° C.; Brett, 1956). Increasing the tonicity of the chilling water exerted no significant effect on the guppy's cold tolerance, as shown by the fact that 21 out of 30 fish chilled in distilled water, 21 out of 30 fish chilled in 0.32 molar sodium chloride, and 20 out of 30 fish chilled in 0.65 molar glucose succumbed.

E. Effect of varying the gaseous content of the test water

1. Effect on mortality of varying the oxygen content and carbon dioxide content of the chilling water (Table II)

Increased $O_2 + CO_2$ or increased O_2 alone, during a cold exposure, lessened the lethality of the exposure (Exps. #1 and #3 Table II). Increased CO_2 alone had no such effect (Exp. #2 Table II). Exp. #4 (Table II) demonstrated that decreased O_2 during a cold exposure increases the lethality of the exposure.

TABLE II

Effect of increased oxygen and carbon dioxide (Exp. #1), increased carbon dioxide (Exp. #2), increased oxygen (Exp. #3), and decreased oxygen (Exp. #4) during cold exposures on tolerance to "primary chill coma"

	Treatment	Test water equilibrated with	O_2 content mg./L.	Number killed	Level of significance
Exp. #1	5° C. for 8 min.	Air	14.1	49 of 59 (83%)	$p \ll 0.001$
		95% O_2 + 5% CO_2	51.1	8 of 55 (15%)	
Exp. #2	5° C. for 10 min.	Air	14	39 of 40 (98%)	$p = 0.7$
		21% O_2 + 5% CO_2	14	38 of 40 (95%)	
Exp. #3	5° C. for 10 min.	Air	13.6	49 of 50 (98%)	$p \ll 0.001$
		100% O_2	51.5	31 of 46 (67%)	
Exp. #4	8° C. for 10 min.	Air	13.3	6 of 40 (15%)	$p = 0.02$
		100% N_2	<0.25	15 to 40 (38%)	

2. Effect on mortality of increased oxygen before, during, or after a cold exposure (Table III)

   Increased environmental oxygen during or after a cold exposure decreased the lethality of the exposure, but increased environmental oxygen before the exposure did not significantly alter this lethality (Table III). There is no significant difference ($p = 0.1$) between increased oxygen during or after a cold exposure (Batches #3 and #4, Table III).

3. Effect of increased oxygen on mortality when given during or after exposures to 2° C.

   Increased oxygen during one-minute cold exposures to 2° C. did not lessen mortality significantly ($p = 0.2$); 20 out of 30 young guppies (6–18 mg.) died when chilled in oxygen-depleted water (< 0.30 mg. O_2/L.), and 20 out of 40 died in O_2-enriched water (50 mg. O_2/L.).

   Twenty minutes in oxygen-enriched water after a three-minute exposure to 2° C. lessened mortality significantly ($p = 0.005$), for 24 out of 25 adult guppies died when replaced in a stock tank (10 mg. O_2/L.) while only 8

TABLE III

Effect of increased oxygen before, during, or after a cold exposure. Batches of 24 young fish (8 to 20 mg.) were chilled at 5° C. for 8 minutes. Batches #1, #2, and #4 were chilled in oxygen-depleted water (less than 0.30 mg. O_2/L.). Batch #3 was chilled in oxygen-enriched water (50 mg. O_2/L.), (Batch #2 was placed in oxygen-enriched water 40 mg.O_2/L.) for 20 minutes prior to the cold exposure and batch #4 for 20 minutes after the cold exposure.

Batch No.	Treatment	Per cent killed	p Value as compared to the control
1	CONTROL (Normal O_2 before and after)	100	
2	Increased O_2 BEFORE cold exposure	96	0.4
3	Increased O_2 DURING cold exposure	21	<0.001
4	Increased O_2 AFTER cold exposure	46	0.001

out of 25 died when they were placed in oxygen-enriched water (40 mg. O_2/L.) of normal temperature prior to return to the stock tank.

F. Significance of anoxia and cold in causing death

1. Tolerance to anoxia at normal temperatures

Three randomly chosen batches of 10 adult guppies each, acclimated to $27 \pm 2°$ C., were placed in oxygen-depleted water at 26° C. (< 0.30 mg. O_2/L.) for different periods of time. Eight minutes of exposure killed none; 15 minutes killed 50%; and 20 minutes killed 100% ($p < 0.001$).

2. Interaction of cold and anoxia (Table IV)

There is a statistically significant difference in number killed between batch #3 and batches #1, #2, and #4 (Table IV). Nine minutes of anoxia prior to 5° C. for one minute (#3) killed significantly more than 5° C. for one minute alone (#1) ($p = 0.02$), 10 minutes of anoxia alone (#2) ($p = 0.005$), or 9 minutes of anoxia after 5° C. for one minute (#4) ($p = 0.05$). This indicated that anoxia before a cold exposure is more lethal than anoxia after a cold exposure.

TABLE IV

Interaction of cold and anoxia in producing death in "primary chill coma." Fish acclimated to 23 ± 1.0° C. for ten days

Batch No.	Treatment	Number killed
	Decreased oxygen (<0.25 mg./L.)	
1	5° C. for 1 min.	2 of 20 (10%)
2	25° C. for 10 min.	1 of 20 (5%)
3	25° C. for 9 min. followed by 5° C for 1 min.	9 of 20 (45%)
4	5° C. for 1 min. followed by 25° C. for 9 min.	3 of 20 (15%)
5	5° C. for 10 min.	18 of 20 (90%)
	Increased oxygen (50 mg./L.)	
6	5° C. for 10 min.	2 of 20 (10%)

When batch #5 (Table IV) was exposed to 5° C. for 10 minutes, a significantly higher number ($p = 0.005$) died than in batch #3 where fish were exposed to anoxia for 9 minutes and then to 5° C. for one minute. There is a highly significant difference between batch #5 and all the other batches. Batch #6 was exposed to 5° C. for 10 minutes in oxygen-enriched water (50 mg. O_2/L.); but only 2 out of 20 died as compared to 18 out of 20 in batch #5 which was exposed to 5° C. for 10 minutes in oxygen-depleted water (< 0.25 mg. O_2/L.) ($p \ll 0.001$).

G. Effect of prior anesthesia with 1% urethan (Fig. 4)

Prior anesthesia with 1% urethan decreases the lethal effect of "primary chill coma" ($p = 0.005$) (Fig. 4).

H. Effect of varying the sequence of two cold exposures (Table V)

Comparing batches #1 and #2 (Table V), a significantly higher number died ($p = 0.001$) among the fish exposed to the colder temperature first.

A suggestive decrease in mortality ($p = 0.1$) to an exposure to 2° C. for one minute occurred if the fish were first exposed to 8° C. for 4 minutes (Batches #2 and #3, Table V).

There is no statistically significant difference ($p = 0.25$) between 2° C. for

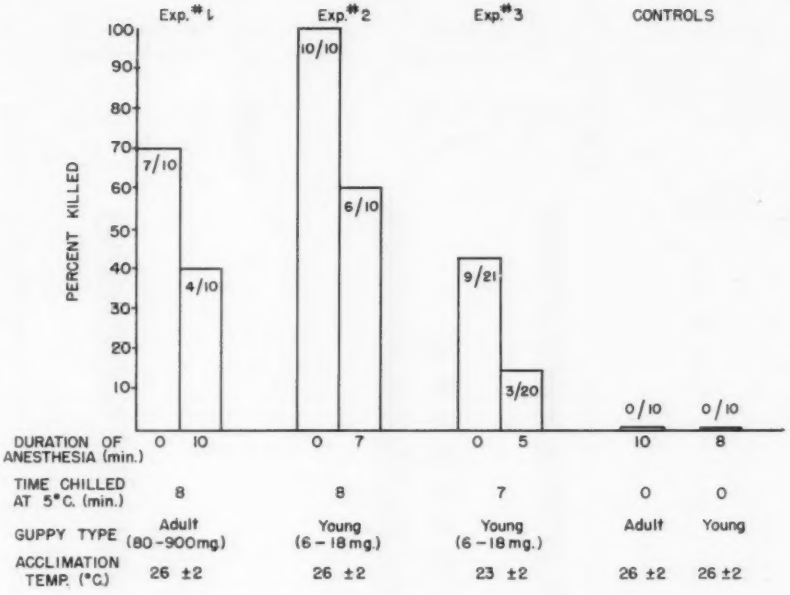

FIGURE 4. Protective effect of anesthesia with 1% urethan immediately before a cold exposure ($p = 0.005$).

5 minutes (Batch #4), and 2° C. for 1 minute when followed by 8° C. for 4 minutes (Batch #1).

There is a profound difference ($p \ll 0.001$) in lethal effect between 2° C. for 5 minutes (Batch #4), and 8° C. for 5 minutes (Batch #5).

II. Secondary Chill Coma

A. Study of a single population (Fig. 5)

1. Effect of the duration of a cold exposure on mortality

At 10.5° C., 20 out of 40 fish exposed for one hour died while 29 out of 40 exposed for one and one quarter hours died ($p = 0.05$). Only two

TABLE V

Effect of varying the sequence of two cold exposures. Three groups of ten adult guppies were used for each treatment

Batch No.	Treatment	Number killed
1	2° C. for 1 min. followed by 8° C. for 4 min.	25 of 30 (83%)
2	8° C. for 4 min. followed by 2° C. for 1 min.	12 of 30 (40%)
3	2° C. for 1 min.	19 of 30 (63%)
4	2° C. for 5 min.	30 of 30 (100%)
5	8° C. for 5 min.	2 of 30 (7%)

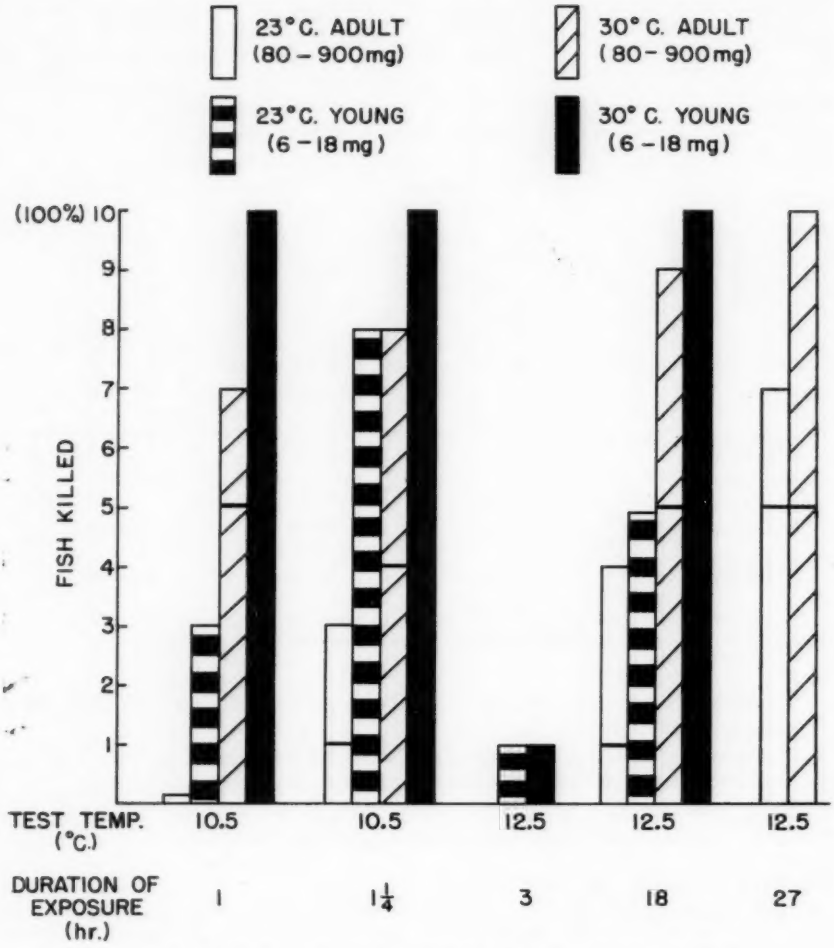

FIGURE 5. Death due to "secondary chill coma" in the guppy. The black bar separates females above from males below.

out of 20 young guppies exposed to 12.5° C. for 3 hours died while 15 out of 20 exposed for 18 hours died ($p < 0.001$). Thirteen out of 20 adults exposed to 12.5° C. for 18 hours died while 17 out of 20 exposed for 27 hours died ($p = 0.25$). (This last comparison is the only one of the three where the difference is not statistically significant.) These data indicate that in "secondary chill coma," as in "primary chill coma," the duration of exposure determines the mortality.

TABLE VI

Effect of increased oxygen content of the chilling water on tolerance to "secondary chill coma." Fish acclimated to 27 ± 2° C. Combining the results from the three experiments, $p = 1.0$

	Treatment	Number killed	
		10 mg. O_2/L.	45 mg. O_2/L.
Adult fish (100–800 mg.)			
Exp. #1	11.4 ± 0.3° C. for 15.5 hrs.	24 of 25 (96%)	25 of 25 (100%)
Exp. #2	12.1 ± 0.2° C. for 8.0 hrs.	15 of 20 (75%)	15 of 20 (75%)
Juvenile fish (20–40 mg.)			
Exp. #3	12.1 ± 0.2° C. for 12.0 hrs.	20 of 25 (80%)	19 of 25 (76%)

2. Effect of sex, size, and acclimation temperature on mortality

Comparing data in Figure 5, males do not seem less cold-tolerant than females ($p = 0.3$); but young are much less cold-tolerant than adults ($p = 0.005$). Cold-tolerance is inversely related to the acclimation temperature in both young ($p < 0.001$) and adults ($p = 0.005$).

B. Effect of increased oxygen during the cold exposure (Table VI)

Increased oxygen during secondary chill coma did not lessen mortality (Table VI).

C. Effect of isosmotic chilling medium on mortality

Two batches of young guppies (8–18 mg.) were subjected to 12.0° C. for 5.5 hours. Batch #1 was chilled in distilled water and batch #2 in 0.16 molar sodium chloride (F.P. − 0.6° C. This is approximately isotonic with teleost body fluids (Brett, 1956). Nine out of 23 fish in batch #1 succumbed; 10 out of 24 in batch #2 died. There is no significant difference between these two batches ($p = 0.75$).

DISCUSSION

Among the possible causes of death inherent in cold exposure, two factors may be excluded. The suddeness of a cold exposure is not of itself lethal, for sudden exposures did not cause more mortality than gradual exposures (IB1)[3] (Britton, 1924). Moreover, the cooling process *per se* is not lethal since even repetitive chilling into "primary chill coma" caused no mortality (IB2). At a specific cold temperature, the duration of cold exposure is the decisive determinant of lethality rather than the abruptness or repetition of the temperature change.

"Primary Chill Coma"

Taken altogether, the data suggest that "primary chill coma" kills by causing anoxic damage to a cold-depressed respiratory center. The permanent re-establishment of respiration after "primary chill coma" is the critical event in determining survival (IC). Even after prolonged lethal exposures to cold, the fish's circulation often returns within a few minutes (IC) and then, depending on one major variable, permanent respiration does or does not return. This variable is the

[3] This refers to pertinent experimental data listed under results.

amount of oxygen which the circulation can bring to the respiratory center to forestall anoxia damage while the cold depression of respiration subsides.

There appear to be several factors limiting the availability of oxygen. Sumner and Doudoroff (1938) showed that fish have a considerable oxygen reserve in their tissues. Consequently, in the present experiments the relative depletion or saturation of the fish's oxygen reserve partially determines the amount of oxygen that the circulation can carry to the fish's respiratory center. Normally the oxygen reserve is probably maximal, since increased oxygen before "primary chill coma" did not lessen mortality (IE2). If the oxygen reserve had been submaximal, then the extra oxygen should have diffused into the fish's superficial tissues (Privol'nev, 1956; Breder, 1941) and protected the animal against anoxia. Any process which depletes the oxygen reserve increases mortality. The oxygen reserve may be depleted by a prior anoxic period (IF2), decreased environmental oxygen during a cold exposure (IE1), or the extra oxygen consumption which accompanies the initial convulsive paroxysm. Anesthesia prior to a cold exposure lowered mortality (IG), probably by lessening the initial convulsive paroxysm and so lessening this initial depletion of the oxygen reserve.

The protective value of increased oxygen during and after chilling (IE) may be explained by the hypothesis that when the fish's oxygen reserve is depleted, more oxygen can diffuse into the fish and replenish that portion of the depleted oxygen reserve located in the cutaneous and subcutaneous tissues. Ultimately the oxygen probably protects by acting centrally. Nervous tissue is the most sensitive to anoxia of any vital tissue, and so it is probable that the increased oxygen benefits the respiratory center directly. This effect of increased environmental oxygen in promoting respiratory return and survival does not seem to be related to any reflex mechanism for at least two reasons: (1) Increased environmental oxygen would lessen rather than increase reflex stimulation of the respiratory center; (2) increased oxygen protected even when given only during the cold exposure and not when respiration actually restarted (IE).

The observation that four of the thirty individually observed fish died after respiration was re-established (IC) may have either of two alternative explanations: (1) There is an additional mechanism or mechanisms for death due to primary chill coma; (2) when rewarmed the cold depression of the respiratory center was relieved and it began functioning while still anoxic; this rendered it more anoxic and permitted irreversible damage to occur.

In experiments where oxygen was unaltered, mortality was increased by lowering the temperature of a cold exposure of a specific duration (IA1) (IH) and also by subjecting fish first to the lower of two sequential cold exposures (IH). The lower chilling temperature probably induces a more profound respiratory depression and a more vigorous initial convulsive paroxysm which more completely depletes the oxygen reserve.

Tolerance to "primary chill coma" is inversely related to acclimation temperature (IA1). Perhaps a higher acclimation temperature lessens the fish's oxygen reserve since there is less oxygen dissolved in body water of higher temperatures; however, this is partially offset by an increase in diffusion (Krogh, 1919). A higher acclimation temperature increases the overall metabolic rate so that the fish's oxygen reserve is depleted more rapidly. With an increase in the acclimation temperature

the central nervous system would have an increased oxygen need (Freeman, 1950) and the respiratory center would become anoxic more rapidly.

Oxygen may be important also in the observations that young are less tolerant to "primary chill coma" than adults (IA1) and males are less tolerant than females (IA1). These less tolerant groups are smaller, have a proportionately higher metabolic rate (Muller, 1942), and so upon chilling might become anoxic more rapidly. Perhaps they would experience also a more profound respiratory depression, for their smaller size would permit the cold to penetrate more rapidly to the respiratory center. Their smaller size should permit a more rapid replenishment of oxygen reserve via cutaneous diffusion, but evidently this advantage is overshadowed by the higher metabolic rate and perhaps by the more rapid penetration of the cold in the smaller fish. It is possible that young guppies and/or male guppies may have a respiratory center which is innately more susceptible to cold depression or anoxic damage or both.

"Secondary Chill Coma"

The experimental data presented in this paper give no indication of the cause of death due to "secondary chill coma." Osmoregulative failure (Doudoroff, 1945) does not seem to occur in the guppy, for chilling in isosmotic sodium chloride had no protective effect (IIC). Although increased oxygen protects against "primary chill coma," it does not protect against "secondary chill coma" (IIB).

Certain observations are perhaps significant. Fish acclimated to higher temperatures are more sensitive to "secondary chill coma" (IIA2); young are more sensitive than adults (IIA2). These more sensitive groups have a significantly higher metabolic rate and would exhaust their energy stores more rapidly so that death due to "secondary chill coma" might be some sort of exhaustion phenomenon. There does not seem to be a sexual difference in sensitivity to "secondary chill coma." The difference in metabolic rate between male and female guppies is perhaps not enough to produce significant differences in the limited number of fish used.

Despite the present inability to explain the mechanism or mechanisms of death due to "secondary chill coma," all the data that have been obtained to the present time can be reconciled with the proposition that death due to "primary chill coma" is caused by anoxic damage to a cold-depressed respiratory center.

I am indebted to the late Dr. L. V. Heilbrunn for first stimulating my interest in this problem and for providing laboratory space. I wish to thank Dr. John R. Brobeck for sponsoring this project and for his many valuable suggestions and encouragement, and Dr. Harold T. Hammel for his advice and aid.

Summary

A. Observations concerning both "primary chill coma" and "secondary chill coma"

1. In guppies acclimated to 23 to 30° C., exposures to temperatures below 10° C. produced "primary chill coma" while exposures to lethal temperatures above 10° C. caused "secondary chill coma."

2. The duration of a cold exposure at a specific temperature is the decisive lethal

determinant rather than the chilling temperature *per se*. An increase in the duration of a cold exposure causes an increase in mortality.

3. Tolerance to a cold exposure is inversely related to acclimation temperature. Tolerance to oxygen lack at normal temperatures also is inversely related to acclimation temperature.

4. Males are less cold-tolerant than females in the temperature range of "primary chill coma." There does not seem to be a sexual difference in cold tolerance in the temperature range of "secondary chill coma."

5. Young guppies (6–18 mg.) are less cold-tolerant than adults (80–900 mg.).

B. Observations on "primary chill coma"

1. Respiration did not return in any fish subjected to approximately three or more times the LD50 cold exposure, despite the fact that the circulation returned in all fish subjected to approximately twelve times the LD50 exposure or less.

2. The lethality of a cold exposure was increased by decreased oxygen before or during the exposure.

3. The lethality of a cold exposure was decreased by prior anesthesia with 1% urethan, increased oxygen during or after the exposure, or with two sequential cold exposures by exposing the fish to the less extreme temperature first.

4. The lethality of a cold exposure was unaltered by increased oxygen before the exposure, decreased oxygen (of a duration which was not lethal of itself) after the exposure, increased tonicity of the chilling water, or equilibrating the chilling water with 5% CO_2.

C. Observations on "secondary chill coma"

1. The lethality of a cold exposure was unaltered by increased oxygen during the exposure, or by the use of isotonic sodium chloride as the chilling medium.

D. The following conclusions may be drawn from the observations made:

1. Death due to "primary chill coma" in the guppy may be due to anoxic damage to a cold-depressed respiratory center.

2. The lethal effect of "primary chill coma" is related to the profoundness of respiratory depression and by the degree of depletion of the fish's oxygen reserve.

3. Osmoregulative failure does not seem to be a cause of death in "secondary chill coma" in the guppy.

4. Oxygen lack is not a lethal determinant in "secondary chill coma" in the guppy.

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FURROWING IN FLATTENED SEA URCHIN EGGS

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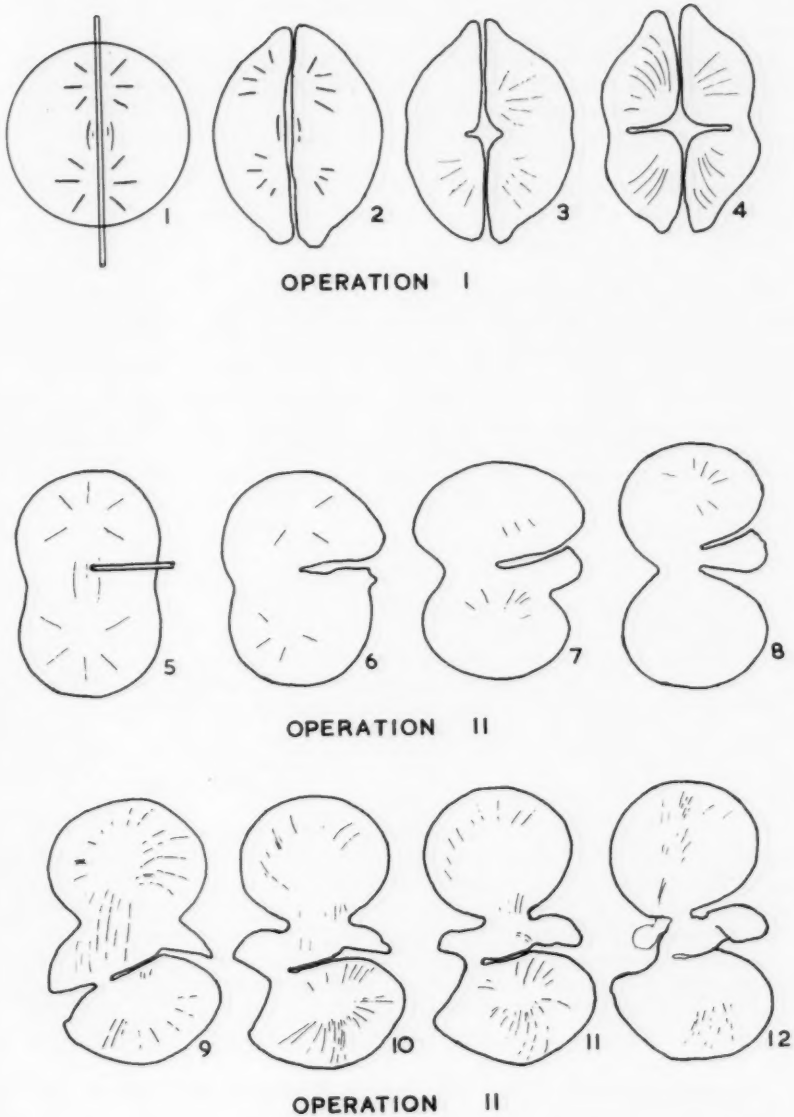
Fifty years ago Yatsu (1908, 1910, 1912) operated with hand-held instruments on cleaving eggs of the nemertine, Cerebratulus and the ctenophore, Beroë. He was able to remove large polar segments from cleaving eggs without interfering with the process. He found that once started in division, the cells demonstrated the greatest pertinacity to divide in spite of operations of the grossest sort. In the years since, many workers have used microdissection techniques on dividing cells: Chambers (1924, 1938) for many exploratory dissections; Dan (1943) to test his astral theory of cytokinesis; Mitchison (1953) in connection with the surface expansion theory; and Hiramoto (1957) to measure the thickness of the cortex during the division cycle. We have used the method to test whether or not "isolated" pieces of furrow cortex undergo contraction. We used the swollen flattened egg as our experimental material because when flattened the mitotic figure is clear, and because cuts can be placed with precision. We have concluded from observations reported here that the position of the furrow is fixed only after elongation has begun, that its position is not necessarily related to the equatorial plane, and that the direction of constriction may be completely altered during the division.

It is not yet possible to adopt any theory of the mechanism of cell division without the gravest reservations. The recent review by Swann and Mitchison (1958) lists eight theories, each one designed to explain a limited set of observations; no over-all theory has emerged, indeed two quite opposite schemes have been proposed to account for the surface changes first observed by Dan, Yanagita and Sugiyama in 1937. According to one of these theories the non-furrow surface expands actively during division (Mitchison, 1952; Swann, 1952); the other theory holds that the furrow cortex contracts actively (Marsland and Landau, 1954). No experiment has yet been reported which will test these two alternatives in a critical way; however, in a previous paper (Scott, 1946), we presented evidence for the continued contraction of the long gelated stalks which occur when eggs cleave in hypotonic sea water. In this paper we present further evidence for the contraction hypothesis.

Material and Methods

Extreme flattening of the sea urchin egg suppresses cytoplasmic division but the division of flattened eggs is facilitated when they are swollen in 60% sea

[1] This investigation was carried out during tenure of a National Science Foundation Fellowship. We are indebted to the Stazione Zoologica, and especially to its Director, for facilities furnished and for the general spirit of helpfulness.



FIGURES 1–12. Operation I, Figures 1–4, hemisection of the entire egg followed by division of each half egg. Operation II, Figures 5–8 and 9–12, near abscission of large polar segments with completion of furrowing.

water. This seems to be due to a more fluid endoplasm which offers less resistance to the constricting furrow.

The eggs of *Paracentrotus lividus* at Naples are approximately 88 micra in diameter. In 60% sea water they swell to a nearly constant diameter of 100 micra in ten minutes and so increase their volume by 47%. If the swollen eggs are flattened to a diameter of 150 micra their thickness is reduced to approximately 30 micra.

The general technique used was as follows: Eggs were obtained from excised ovaries and were washed twice by settling and decantation. Since there is great variability in the ripeness of different females, tests were made to find a female whose eggs produced high fertilization membranes in at least 95% of the eggs sampled. If control samples of any egg batch failed to cleave with at least 95% normal first cleavage, the egg batch was discarded. The fertilization membranes were removed at three minutes by shaking. At 65 to 70 minutes the eggs were removed to 60% sea water and prepared for microdissection in a hanging drop on a very clean, previously flamed, slide. The degree of flattening was regulated by withdrawing the appropriate amount of the dilute sea water with a fine pipette. Most of the operations were slit punctures made with the flat side of a microneedle. Observations were made on the flattened eggs still mounted in the moist chamber. The manipulator was made by De Fonbrun and the needles were drawn with a De Fonbrun microforge.

Results

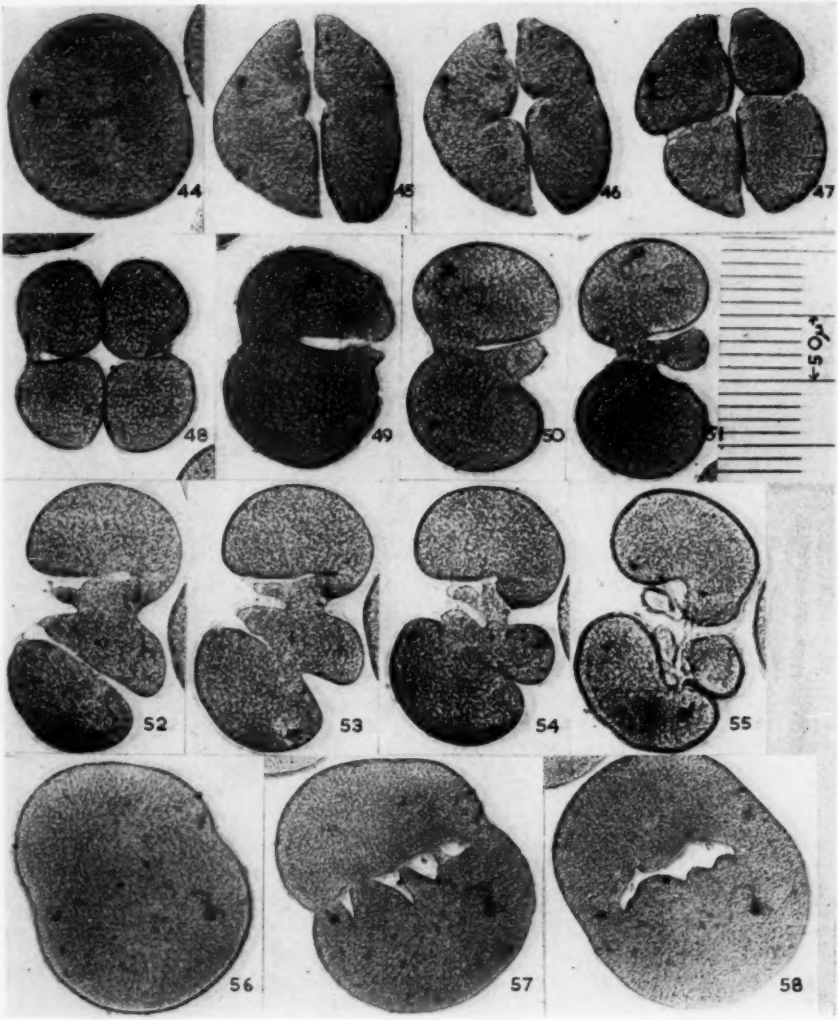
Ten different experiments were performed on the flattened egg. The operations reported involve only the period from mid-diaster, that is, about ten minutes before the first cleavage begins, through the furrowing period. The ten operations are designated I through X and are individually described in the following paragraphs and figured in the accompanying line drawings, Figures 1–43 and photographs, Figures 44–80.

Operation I, hemisection of the entire egg, Figures 1–4 and 44–48:

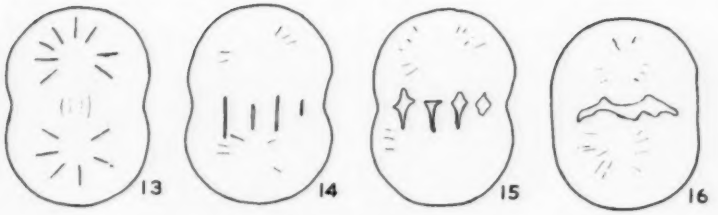
It is surprising to find that when eggs are bisected with a cut which divides the amphiaster neatly through its long axis, both fragments cleave. Each fragment retains visible parts of the half asters and the half spindle. Sometimes the furrow cuts in symmetrically towards the new median longitudinal axis of the half egg or alternatively it cuts out from the plane of hemisection. Yatsu reported that large segments cut from the "side" of the dividing egg completed the furrowing but this observation of cleaving hemisected eggs has a certain elegance when performed on a flattened egg, in that the flatness allows a precise bisection of egg and amphiaster.

Operation II, abscission or near abscission of polar segments, Figures 5–8, 9–12, and 49–55:

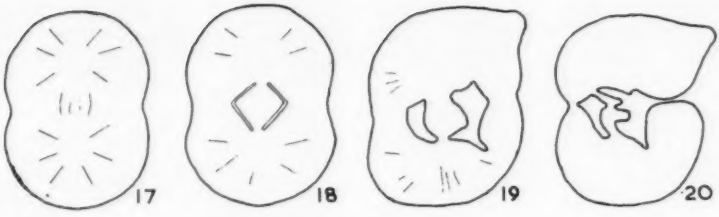
It is possible to cut away one or both poles at the cylinder stage, removing as much as two-thirds of the egg substance, and still get successful furrowing. Neither the position of the furrow nor its course is altered by the operation. This experi-



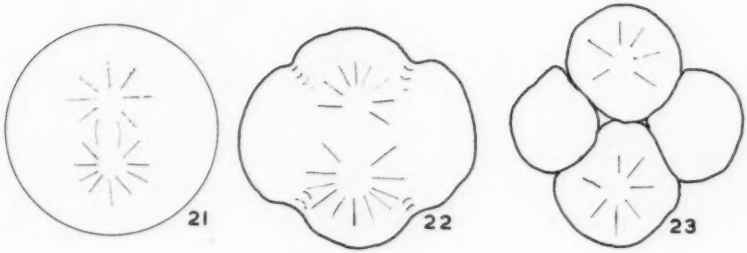
FIGURES 44–58. Photographs illustrating Operations I–III. Operation I, Figures 44–48, hemisection of entire egg with continued furrowing. Operation II, Figures 49–51, continued furrowing after near abscission of one pole. Operation II, Figures 52–55, continued furrowing after near abscission of both poles. Operation III, Figures 56–58, rapid opening of slit perforations (see text). Figure 57 is a few seconds after Figure 56; Figure 58 is 5 minutes later.



OPERATION III



OPERATION IV



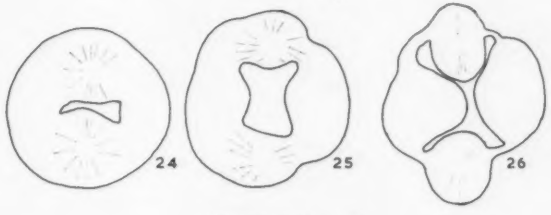
OPERATION V

FIGURES 13–23. Operation III, Figures 13–16, longitudinal slits in the furrow showing the immediate opening-out of the slits and the completion of three internal furrows. Operation IV, Figures 17–20, near isolation of a furrow segment, Figure 18, followed by shrinkage of the segment, Figures 19 and 20. Operation V, Figures 21–23, cleavage of a flattened diastral egg to four cells; two cells with, and two without, aster and nucleus.

ment separates the expanding polar surface from the rest of the egg, during a considerable part of the time that it would be expanding in the unoperated egg, without interfering with cleavage.

Operation III, longitudinal slits in the furrow, Figures 13–16 and 56–58:

During this operation, the egg is flattened in the cylinder stage and one or more slit perforations are made across the furrow with the long axis of the slit parallel

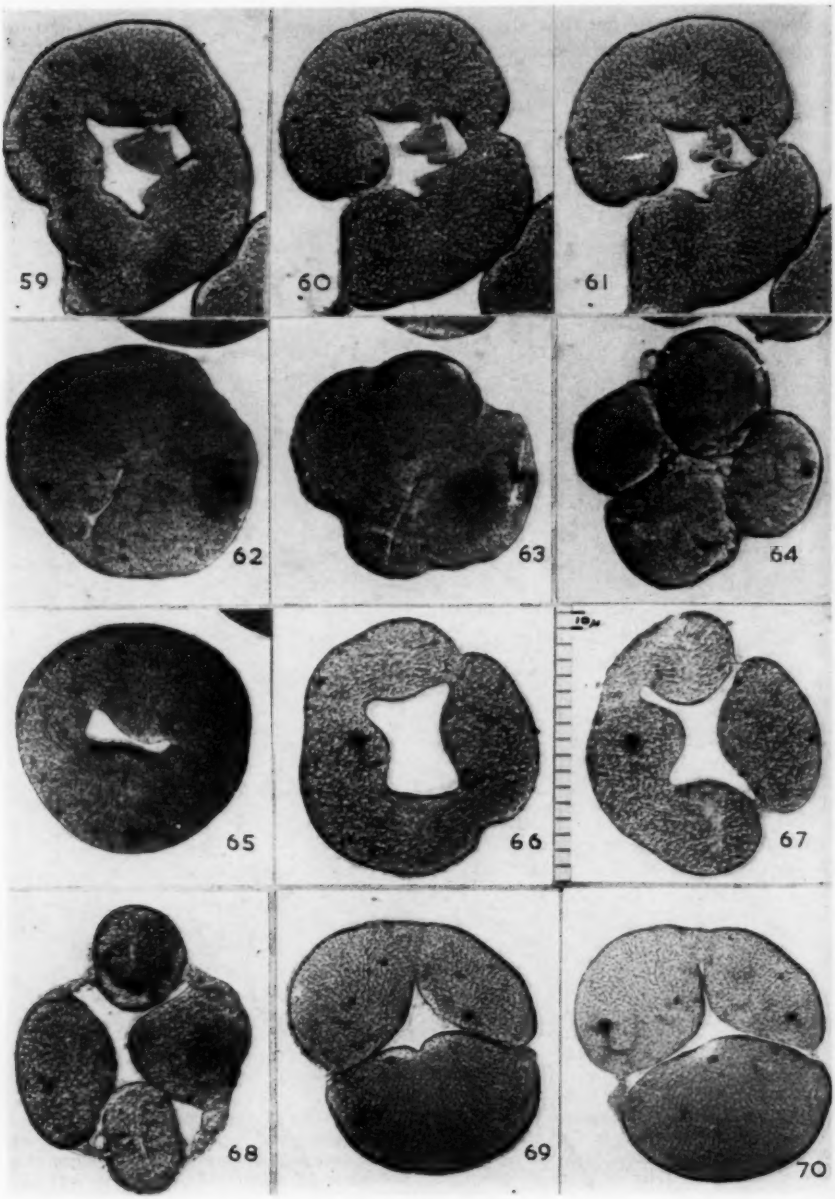


OPERATION VI

OPERATION VII CASE I

OPERATION VII CASE II

FIGURES 24–33. Operation VI, Figures 24–26, transection of the spindle; development of four furrows and cleavage to four cells, two cells without asters and without nucleus. Operation VII, Case I, Figures 27–29, slit perforations lateral to the spindle; five furrows produce four cells, two without aster or nucleus. Operation VII, Case II, Figures 30–33, slit perforations lateral to the spindle; three cells are formed, one without aster or nucleus.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to the long axis of the egg. The slits immediately open in a way that gives evidence that the furrow ring is a region of tension in flattened eggs. Within seconds the slits open and multiple furrows produced gradually cut through the narrowing stalks. This operation makes cleavage possible in eggs that would otherwise be stalled at the cylinder stage; it is apparently easier for an egg to furrow through several small stalks than through one big one.

Operation IV, near isolation of a furrow segment, Figures 17–20 and 59–61:

In this operation four perforating cuts are made as shown in Figure 18, so that a central plug of egg substance, the surface of which is virtually all furrow cortex, is isolated from the rest of the egg. Only slender stalks connect the plug with the sub-furrow region. During the next few minutes the whole semi-isolated plug shrinks in volume; meanwhile its contained endoplasm flows through the attachments into the main part of the cell. We consider this to result from an active contraction of the entire surface of the plug; it appears to be incompatible with the membrane expansion theory.

Operation V, flattening without microdissection, Figures 21–23 and 62–64:

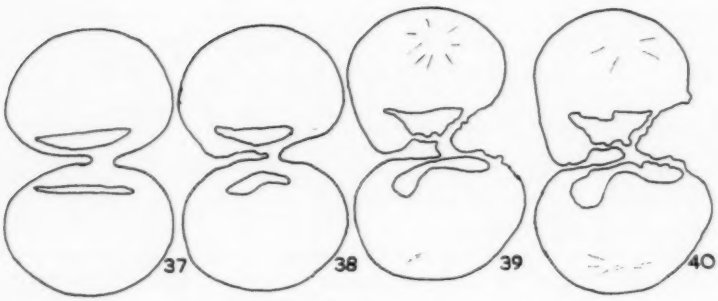
When eggs in the middle diaster are swollen, flattened but otherwise unoperated, they may cleave directly to four cells, two of which contain asters and nuclei and two that do not. The expected single equatorial furrow does not develop; instead, four areas of furrowing move in from the periphery following the tips of the astral rays. In two cases the four-celled embryo was followed through a second cleavage during which the nucleated cells divided again while the anucleate ones failed to divide; the latter did, however, each develop a large monaster during the "division phase," which is the more interesting since they were patently devoid of any part of the amphiaster during the first division.

Operation VI, equatorial transection of the spindle I, Figures 24–26 and 65–68:

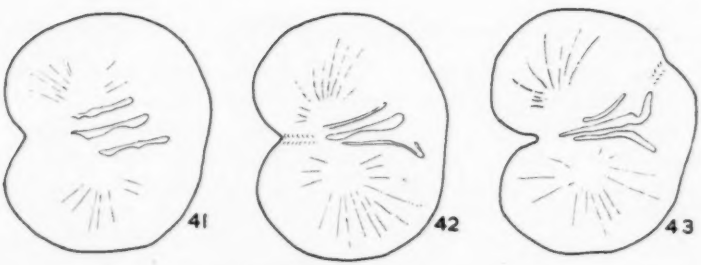
When one makes a slit-perforation across the equator of the spindle of a mid-diaster stage the perforation soon opens up to form a large hole. The egg then divides directly to four cells, two with nuclei and two without. This is not an occasional result but occurs quite regularly following the transection operation. In all cases with central perforations the furrowing tends to be centrifugal; the furrow cuts out from the hole.

FIGURES 59–70. Photographs illustrating changes after Operations IV, V, VI, and VIII. Operation IV, 59–61, isolation of an island of furrow substance at the cylinder stage showing shrinkage of the furrow island. Operation V, Figures 62–64, flattening without dissection of the furrow with subsequent division of the diastral cell to four cells; two with aster and nucleus and two without. Operation VI, Figures 65–68, transection of spindle of a diastral cell followed by cleavage to four cells; two with an aster and a nucleus and two without. Operation VIII, Figures 69 and 70, bisection of one aster with continued furrowing in the furrow plane but no furrowing between the halves of the divided aster.

OPERATION VIII



OPERATION IX



OPERATION X

FIGURES 34–43. Operation VIII–X, Figures 34–36, bisection of an aster; each half aster forms a spherical aster. Operation IX, Figures 37–40, isolation of walls of completed furrow by means of slit perforations. The tubes of cortex thus formed subsequently shrink in diameter. Operation X, Figures 41–43, isolation of the early furrow cortex by means of slit perforations across the spindle axis. The cortical tubes formed shrink in diameter. Figure 41, immediately after the operation; Figure 43 five minutes later.

Operation VII, slits lateral to the spindle, Case I, Figures 27–29 and 71–73:

Four daughter cells are formed when paired perforations are made on opposite sides of the amphiaster. First there is a furrowing across the spindle equator; then four non-spindle furrows work out centrifugally from the ends of the perforations. In this case as in Operation VI two of the four daughter cells are anucleate.

Operation VII, slits lateral to the spindle, Case II, Figures 30–33:

In some cases three furrows form instead of four, producing two cells with nuclei and one without.

Operation VIII, subdivision of an aster, Figures 34–36 and 69–70:

One or both asters may be bisected with a perforating cut which, if placed as indicated in these figures, will have its inner end drawn out in the furrowing. The half asters become asters of smaller size when bisected and each one has a center from which rays diverge more or less symmetrically. All slits made in the vicinity of asters conform to the sphericity of the aster; apparently even subdivided asters tend to be spherical structures.

Operation IX, "isolation" of late furrow cortex, Figures 37–40 and 74–77:

It is possible to isolate the walls of the completed furrow from the rest of the egg by making two perforations across the spindle axis close to the furrow; this operation forms the furrow walls into tubes which shrink in diameter in the same way as the cleavage stalk.

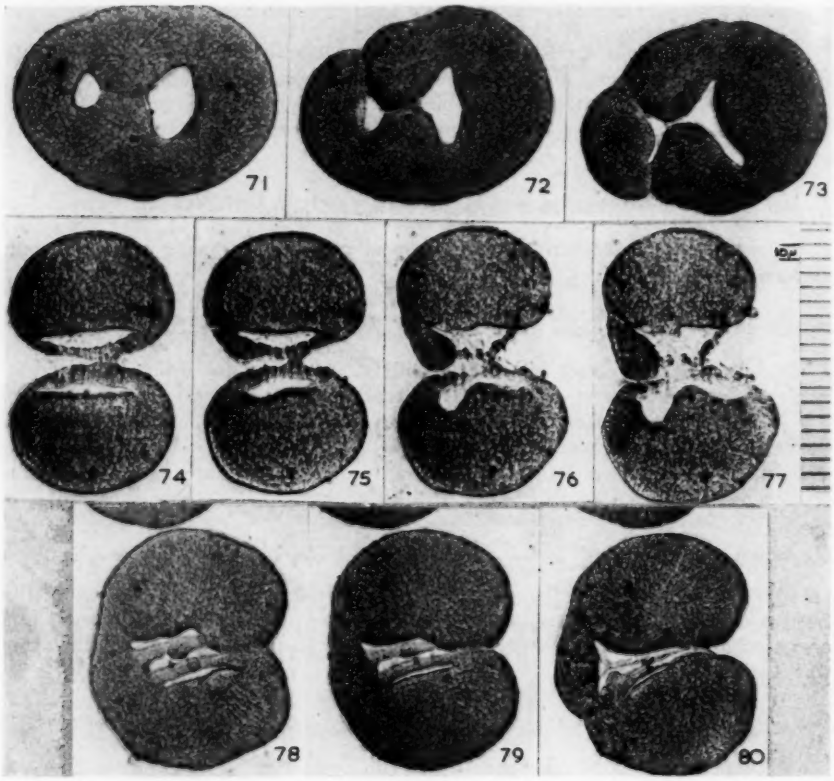
Operation X, "isolation" of the early furrow cortex, Figures 41–43 and 78–80:

In this case the eggs used had just begun to cleave. Two or three slit perforations placed transversely across the mid region isolate tubes of furrow cortex which diminish in diameter like furrow stalks, but their direction of constriction is quite different from the presumptive one.

Discussion

Contraction

We have taken the position that the weight of evidence now favors the theory that cell cleavage occurs by active, cortical contraction. The "proof" will be more compelling if a contractable substance can be extracted from the cell cortex as it can be from muscle. Indeed such proof may be near, for Hoffmann-Berling (1954, 1960) has reported the extraction of a contractile protein from fibroblasts and from sarcoma cells which may be responsible for the furrowing *in vivo* and in his glycerine-water-extracted telophase cell models. One of the strongest indications that the cortex contracts is the shrinkage of the "furrow islands" reported in this study which, although isolated early in furrowing, shrink progressively to become thin strands. In other experiments we produced tubes of furrow cortex



FIGURES 71–80. Photographs illustrating operations VII, IX, and X. Operation VII, Case I, Figures 71–73, slits lateral to the spindle; five furrows form four cells, two with aster and nucleus and two without. Operation IX, Figures 74–77, isolation of tubes of late furrow cortex followed by shrinkage of the tubes. Operation X, Figures 78–80, isolation of tubes of early furrow cortex followed by shrinkage of the tubes.

by a number of different operations (*cf.* Fig. 18 and Fig. 59), after which only narrow connections joined the tubes to the main mass of the egg, and in all cases the tubes diminished in diameter in the manner of a cleavage stalk. Such phenomena are difficult to explain by the membrane expansion theory (Mitchison, 1952; Swann, 1952) which hypothesizes that the expansion of non-furrow surface causes the shrinkages of the furrow. In our experiments furrow cortex is nearly separated from the non-furrow surface, yet it shrinks very effectively. One could argue that the expansion process reaches into the furrow itself but this view must be discarded, as Swann and Mitchison (1958) have pointed out, because there is no area of expansion in the furrow (Dan and Ono, 1954).

Furrow tension

Our experiment III indicates that a circumferential tension is present around the furrow ring by the time of cell elongation. This is contrary to measurements with the elastimeter (Mitchison and Swann, 1955), for no measurable differences in stiffness were found over the surface of the egg at the cleavage stage. We note here that while the eggs used in our studies were swollen, flattened, and certainly abnormal in many ways, still, they did cleave and almost certainly by the same mechanism as normal eggs. Tension around the equator is, as Mitchison and Swann have pointed out, inconsistent with the expansion theory. In their consideration of the flattened egg they have assumed (Mitchison and Swann, 1955) that the tension produced by flattening disappears before expansion begins. They suggest that this would explain the slower furrowing observed after flattening. In the case of experiment III, however, the tension seems never to fall to "zero," for each slit of a series, made one after the other, opens quickly within seconds, indicating that tension still exists around each smaller furrow. The failure to observe tension differences at cleavage with the elastimeter may perhaps be due to the fact that the endoplasm is a fairly viscous gel which resists being drawn into the instrument. Real differences in the stiffness of the cortex would then be masked. It was found that the general stiffness of the surface increases while the asters are quite small, indicating that the aster is not then affecting the measurement, but the "stiffness" value of thixotropic endoplasm is difficult to assess.

What determines where a furrow will form?

In these experiments we have utilized two stages of division for the most part, the one a mid-diaster stage and the other about ten minutes later, at the beginning of cell elongation. The evidence supports the view that the furrow cortex is unspecified during the earlier period and that a definite band of furrow cortex, with special properties, is determined from the moment of cell elongation. It is not possible to perform operations on elongated eggs which will provoke furrowing in areas outside the presumptive furrow. The furrowing cortex is therefore determined; it can be notched, isolated in islands and variously perforated, but it retains its capacity for involvement in constriction. The position of the furrow is not fixed at mid-diaster since simple spindle transection, for example, produces three or four furrows in regions quite different from the presumptive one. What, then, determines where a furrow will form? It has been almost universally accepted that the position of the furrow is related to the spindle equator; thus polar body formation involves such a relationship, whatever the size of the two cells produced, as does micromere formation, the unequal first cleavage in mollusks and abnormal cases involving multipolar spindles. Now, however, the idea of coincidence of mid-spindle and furrow must be abandoned. In our view furrow formation involves three factors:

1. The presence of an aster (not necessarily two), since furrows always follow an astral boundary.

2. The recognition by the cell of a region of narrowness. Deformed cells (see Figure 67) are curiously "opportunistic" in their ability to exploit a region

of narrowness as a potential furrow path. Perhaps the surface in these isthmian regions is differentially stretched and differentially sensitive to furrow initiation.

3. The presence of some (chemical) initiator which may affect the entire circumference of the potential furrow or which may be limited initially to one region of the circumference acting as a furrow head. It seems necessary to assume that the old concept of "furrow head" (Ziegler, 1898) has some validity since many cases occur in which the furrowing process moves progressively over the surface. Yatsu, for example, demonstrated (1910) that the furrow head would bifurcate after some operations disturbing its presumptive path. We conceive of the furrow head in some of these operated eggs as a sector of contraction involving only a part of the furrow ring, the other sector being passive at first, then progressively involved as contraction spreads around the furrow. We conceive, furthermore, of the whole furrow as being under tension as soon as any part of it contracts. This would explain the deep incursions which occur on one side of some furrows while the other side is barely indented. The problem is to explain how an initiator substance appears at precisely those places where furrows will form, for example, at the ends of the slits in Operation III.

Orientation of contractile units

We have shown that operations on the elongated egg may change the orientation of furrowing in areas where it has already begun. We have elsewhere noted other cases in which new directions of furrowing set in (Scott, 1946), for example, in the late furrowing of dispermic eggs where the plane of constriction around the neck of a one-quarter blastomere is displaced 45° from the original plane. In the course of another study, as yet unpublished, we observed changes in the patterns made by echinochrome granules located in the presumptive furrow cortex of cleaving *Arbacia punctulata* eggs. We noted that the furrow surface shrinks two-dimensionally during the earliest phases of the cleavage, from the sphere to the cylinder stage, after which it shrinks only around the circumference of the furrow. We speculate, therefore, that the contractile elements of the furrow cortex are arranged at random in the plane of the surface but that effective contraction can only occur in directions where resistance to shrinkage is low. According to this interpretation the contractile elements of the band of furrow cortex are arranged at random in the plane of the surface; during the sphere-to-cylinder stage they all contract whatever their orientation, but during the subsequent stages of the furrowing only those elements oriented around the furrow are able to shorten. This hypothesis can obviously be extended to include the unusual "furrow" patterns resulting from micromanipulation, for example Figures 74–77 and 78–80. In these latter examples, cylinders of furrow substance shrink in directions quite different from the presumptive ones.

Summary

1. Eggs swollen in 60% sea water cleave effectively when flattened.

2. Furrowing continues after abscission of one or both poles of the egg.

3. Furrowing is initiated and completed after hemisection of the still spherical egg through the long axis of the amphiaster.

4. Bisection of the spindle just before egg elongation causes a diastral egg to cleave into four blastomeres, two of which lack asters.

5. Islands of furrow cortex "isolated" at early furrowing shrink progressively during division. This is regarded as evidence for active furrow contraction.

6. Tubes of furrow cortex prepared from the furrow walls shrink in directions quite different from the original one.

7. The furrow cortex is not determined until the beginning moment of elongation for cleavage.

8. The position of the furrow is not necessarily related to the plane of the spindle equator.

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SURFACE CHANGES DURING CELL DIVISION

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The general features of surface change during cell division have been known for the sea urchin since the inspired work of Dan and his colleagues (Dan, Yanagita and Sugiyama, 1937). Dan's original observations have been substantially confirmed by Hiramoto's excellent analysis (1958). In the present paper we, too, confirm Dan's original observations, although we cannot support his theoretical interpretation (Dan, 1943). The Japanese workers made use of adherent clay or carbon particles to mark the surface; we have used, instead, the echinochrome granules of the egg cortex as natural markers. The detailed behavior of these intensely red granules is of interest because they are fixed in the gelated cortex and there is no possibility of slippage between the surface and the marker. Photographs of the granule patterns give a faithful record of large areas of the cell surface in a way that a few adherent particles can not do. It should be noted that Dan also used the echinochrome granules to mark the movement of the cortex in cleavage but with another aim in view and at much lower magnifications (Dan, 1954). We describe in this paper the surface behavior of a few eggs with some care. The photographs show a "new view" of furrow and pole during division and give especially clear evidence of the polar surface change. The drawings were prepared in every case by tracing photographs.

The term "pole" as used here refers to points on the egg surface normally intersected by the spindle axis. The furrow is considered to have the cell-equatorial plane through its middle. Latitudes are parallel to the equator and longitudes run from pole to pole.

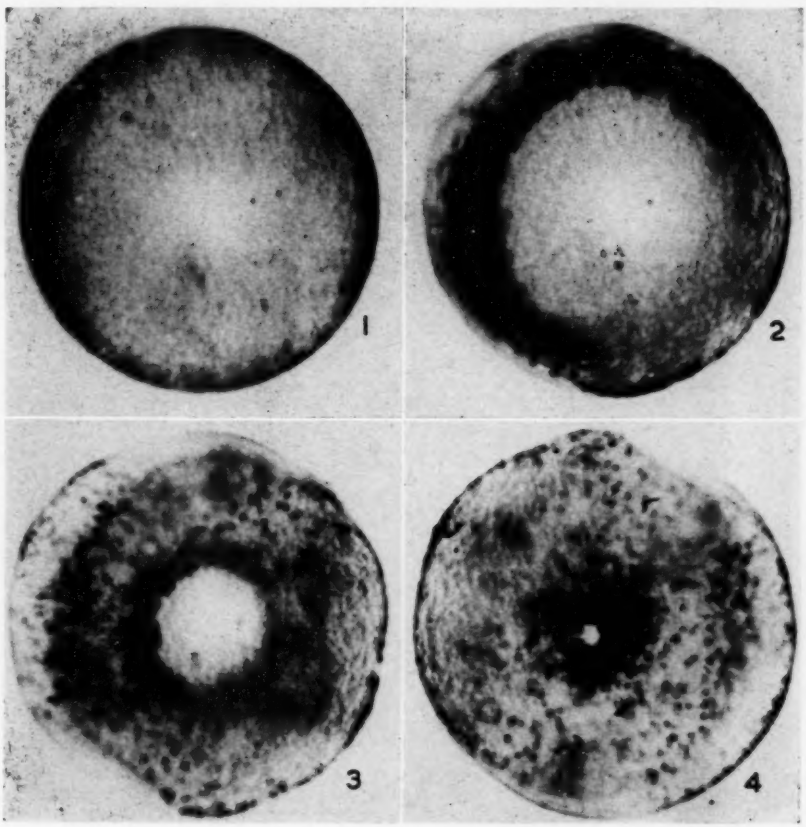
Material and Methods

The red echinochrome granules of the *Arbacia punctulata* egg stand out in strong contrast in the green light of a Wratten No. 58 filter. The individual granules can be photographed sharply through substantial thicknesses of cytoplasm, so that both the surface granules and those deep inside the egg can be seen clearly. The eggs of some females have much darker granules than those of others and, moreover, egg batches differ in the average size of the granules and in the average number of granules per egg.

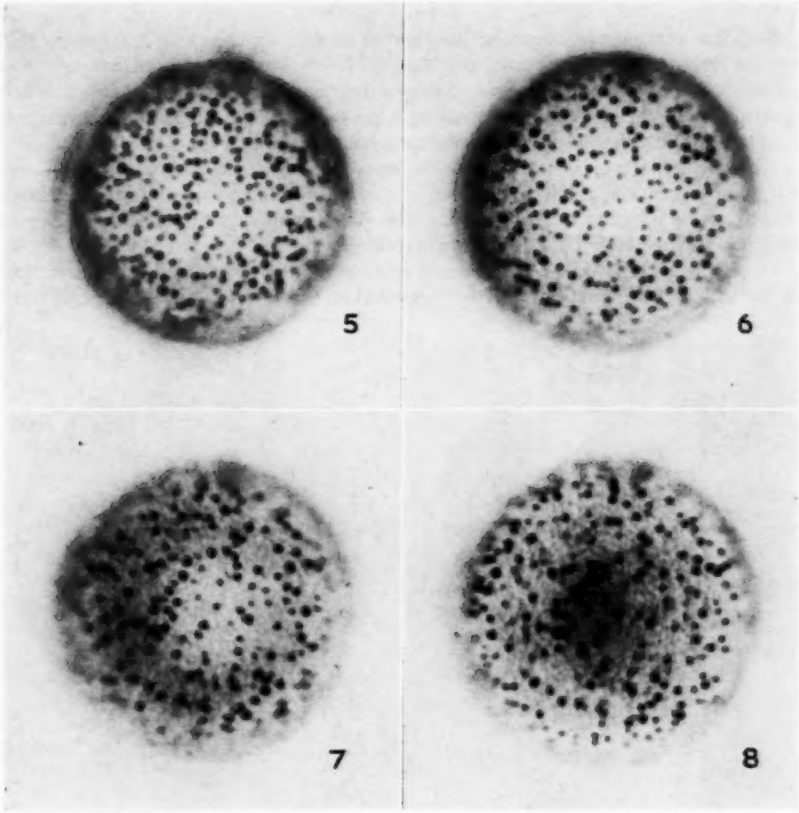
Eggs and sperm were obtained by the electrical method (30 volts A. C.). All of the egg batches used showed at least 95% of the eggs with high fertilization membranes and at least 95% cleavage of the fertilized controls. The fertilization membranes were removed three minutes after insemination by shaking. Just before the cleavage was due, the eggs were transferred to a cold stage held at about 10° C. Cold dry air was blown across the cold stage to prevent condensation; with this apparatus it was possible to take sequential photographs of the same

region of the surface showing the granule patterns in sharp focus. Photographs of the polar surface and views of the furrow seen from the polar axis were taken with the microscope in horizontal position. The eggs were mounted between cover and slide and sealed with Vaseline. When the microscope was tilted into the horizontal position, the eggs came to rest on a glass hair. An occasional cleaving egg was found to be oriented with its long axis coincident with the optical axis. With this technique one polar surface and the equatorial (furrow) plane were observed with good resolution. The inner furrow margin was seen to close like an iris diaphragm, as in Figures 1, 2, 3 and 4. The division of quarter blastomeres can be viewed in this aspect on the upright microscope.

Polyspermic eggs show multiple and intersecting furrows during the first cleavage, in which the regions of furrow intersection show characteristics not found in



FIGURES 1–4. Views of the equatorial plane from the polar axis, showing the iris-like closure of the furrow ring.



FIGURES 5–8. Views of one polar surface of the egg pictured in Figures 1–4. The following pairs of photographs were taken within a few seconds of each other: 1 and 5; 2 and 6; 3 and 7; 4 and 8. Figures 1 and 5, cylinder stage. Figures 4 and 8, at the end of furrowing.

normal, diastral cleavage. Polyspermy was induced by methods detailed previously (Scott, 1946).

RESULTS

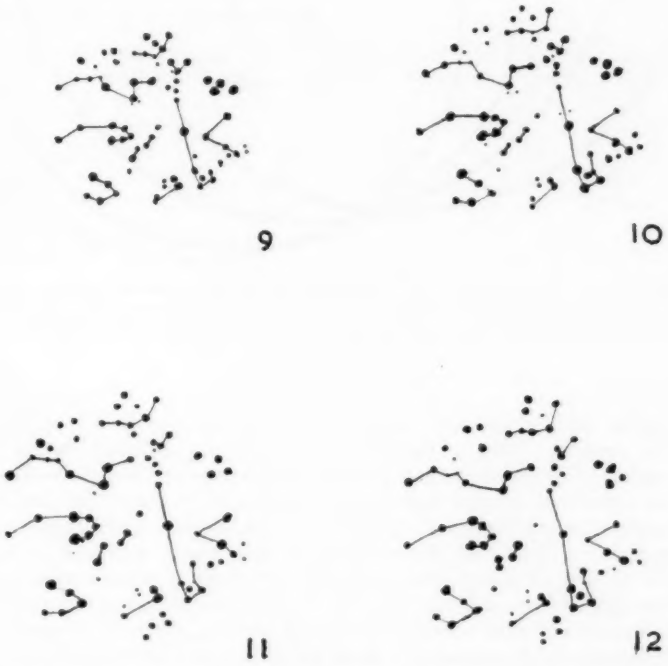
Cortical stiffness indicated by granule patterns

The movements of the echinochrome granules are as follows: (1) In the unfertilized egg they are scattered throughout the cytoplasm; within seven minutes after fertilization at 20° C. most of them have migrated to, and become fixed in, the cortex. (2) The individual granules when observed with oil immersion are seen to undergo a slow random migration within the cortex up until the moment of cell

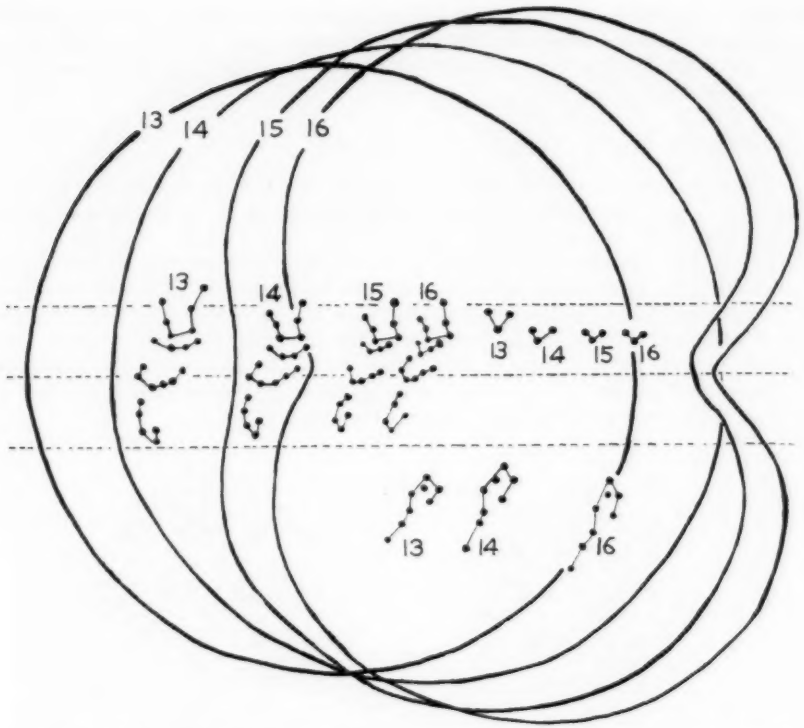
division. (3) During furrowing the cortex is gelated enough to hold the granules in relatively fixed positions so that patterns persist until the end of furrowing when the cortical gel softens and the patterns blur as the granules resume their slow random movement.

Polar surface

The polar surface stretches during furrowing as is illustrated in Figures 5, 6, 7 and 8. The polar surface expands during the first part of the furrowing but remains stretched without further expansion from the time the inner furrow diameter is about 10 micra to the end of the process. The amount of increase in area of the polar zone shown in the photographs is in excess of 65% and the degree of radial expansion is essentially the same for any sector of the polar surface. Constellations of granules have been connected by lines in drawings made from the photographs above (Figs. 9, 10, 11 and 12). In some eggs the expansion continues until the end of furrowing, while in others the expansion is finished by the time the inner furrow diameter reaches ten micra.



FIGURES 9–12. Drawings made from photographs 5–8; the constellations of granules have been connected by lines to make the granule patterns more evident. Polar expansion occurs during the interval between 9–11. There is little change in the polar surface area in late furrowing (compare Figures 11 and 12).



FIGURES 13–16. Egg outlines and corresponding surface granule patterns during four stages of cleavage from the sphere stage to early indenture. Patterns shrink two-dimensionally in Figures 13–14 but only in directions around the furrow in Figures 15 and 16.

Furrow surface

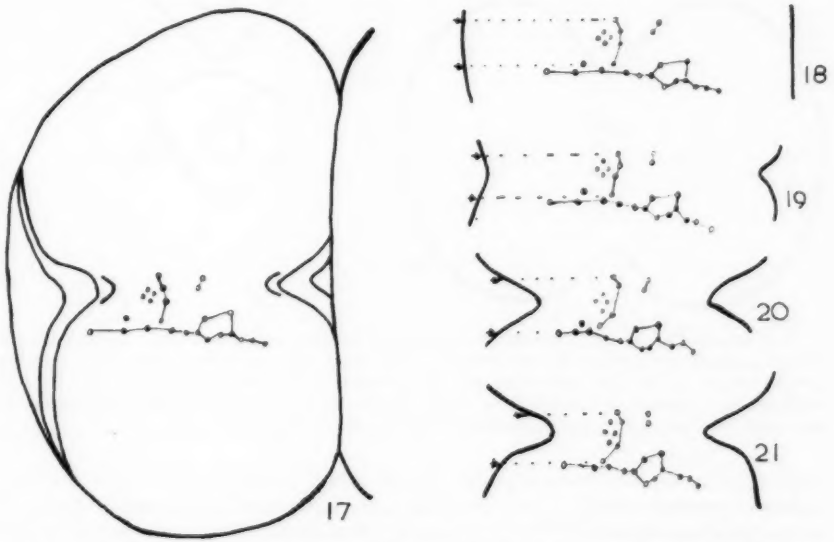
During the earliest phase of cytokinesis the presumptive furrow region flattens around the equator. This is the first phase of surface shrinkage in the potential furrow region. Equatorial shrinkage starts even before cell elongation is apparent. Figure 13 represents an egg just before cleavage is to start. It shows a wide granule pattern on one region of the future furrow surface, while Figure 14 shows the same region a little later when the egg is still nearly spherical. Comparison of Figures 13 and 14 shows that equatorial shrinkage is two-dimensional at this stage in the plane of the surface. The furrow area shrinks along lines of longitude as well as around latitudes. At least one-tenth of the egg surface is surely involved in this shrinkage which represents a band about 15 micra wide around the equator. A longitudinal expansion of the furrow begins at the cylinder stage (Figs. 15 and 22) but its extent can only be appreciated when granule positions are projected to the furrow margin.

Views of the exact center of the furrow show the plastic deformation of the sur-

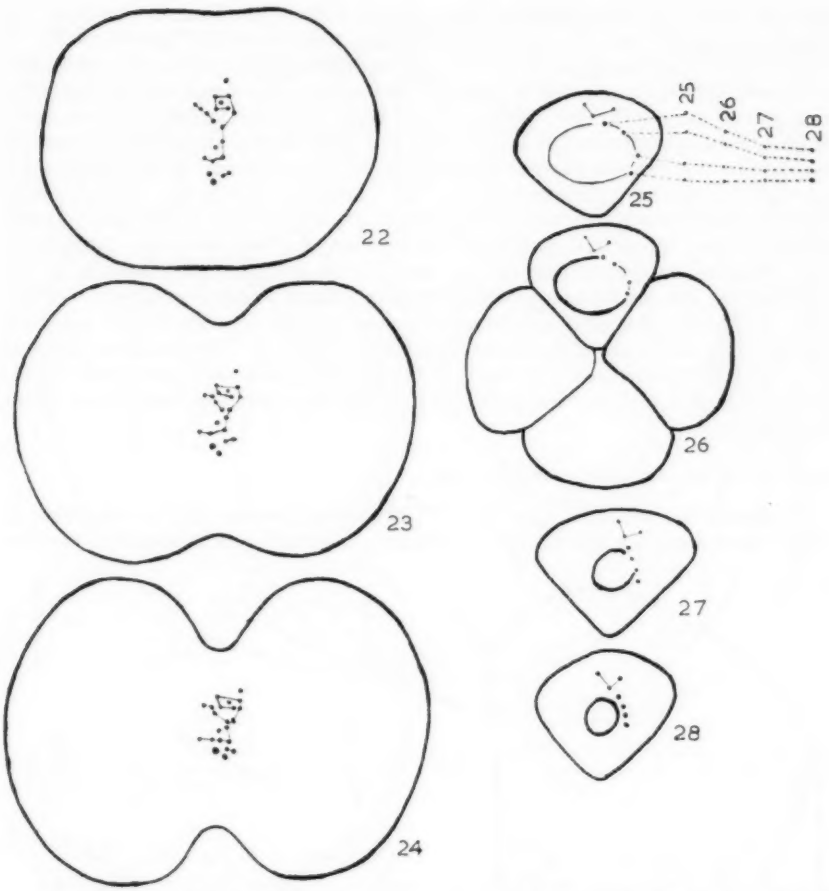
face that occurs in that region. Granule patterns fixed there are deformed by the narrowing equator and by extension in the long axis shown in Figures 17–21. A circumferential shrinkage of the furrow continues throughout cytokinesis, shown also in Figures 17–21, where a row of granules undergoes a steady proportional reduction in length as the cell divides. When viewed from the pole, the furrow closes down like an iris diaphragm, and it is clear that a circumferential shrinkage is occurring throughout this process because granules located on the inner furrow margin are crowded together in a close row as the "iris" boundary moves in. The shrinking circle of granules is clearly seen in Figures 25–28, which illustrate the inner furrow margin in one blastomere during the third cleavage. The successive positions of four granules are diagrammed in four stages of the division. We observe here a shrinkage of this arc to be approximately proportional between each pair of granules. Thus the shrinking substance, whether actively contracting or passively compressed, is shrinking uniformly around the furrow. The granules noted in this case fell behind in the final stages of the furrowing and the furrow peak pushed on beyond them. Shrinkage ends earlier in paraequatorial latitudes than in the equator proper.

Intersecting furrows in polyspermic eggs

Triastral and tetrastral eggs involve intersecting furrows, and at the points of intersection there is a special area of surface whose fate is different from the rest.

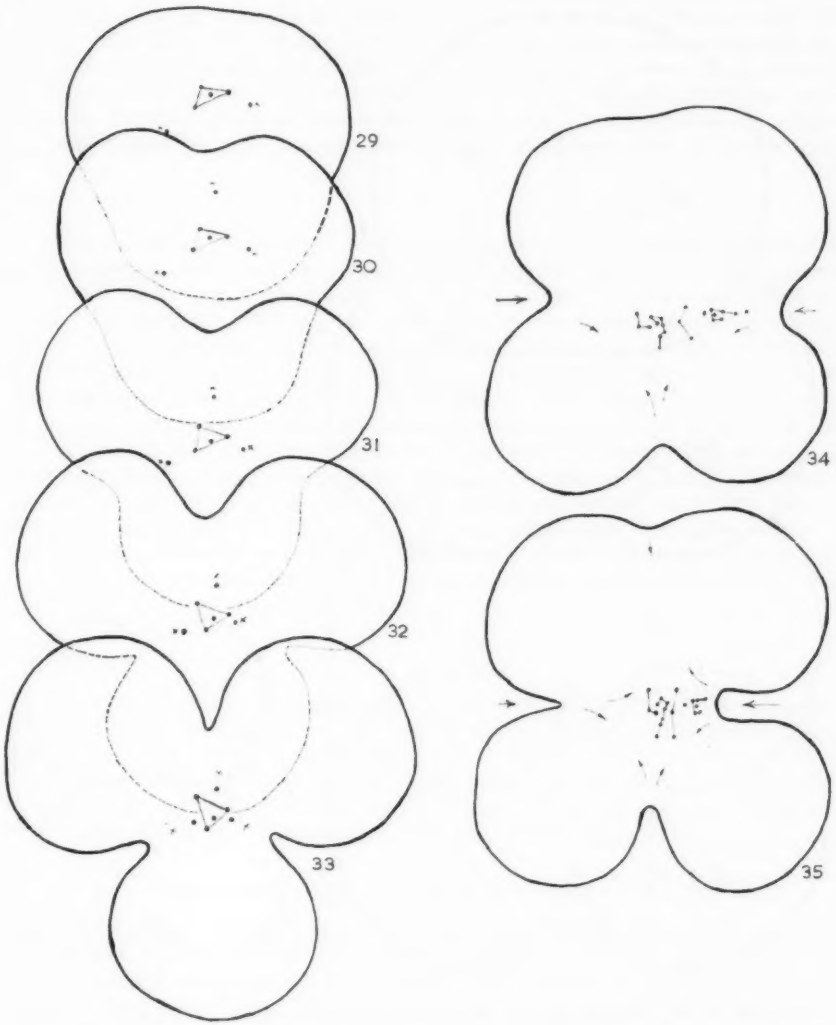


FIGURES 17–21. Granule patterns in mid-furrow and sub-furrow. FIGURE 17. One egg at second cleavage, showing the location of granule patterns in the furrow area. FIGURES 18–21. Details of stages of division in the egg shown in Figure 17, showing circumequatorial shrinkage of patterns on the sub-furrow surface and plastic deformation of patterns at the furrow peak.



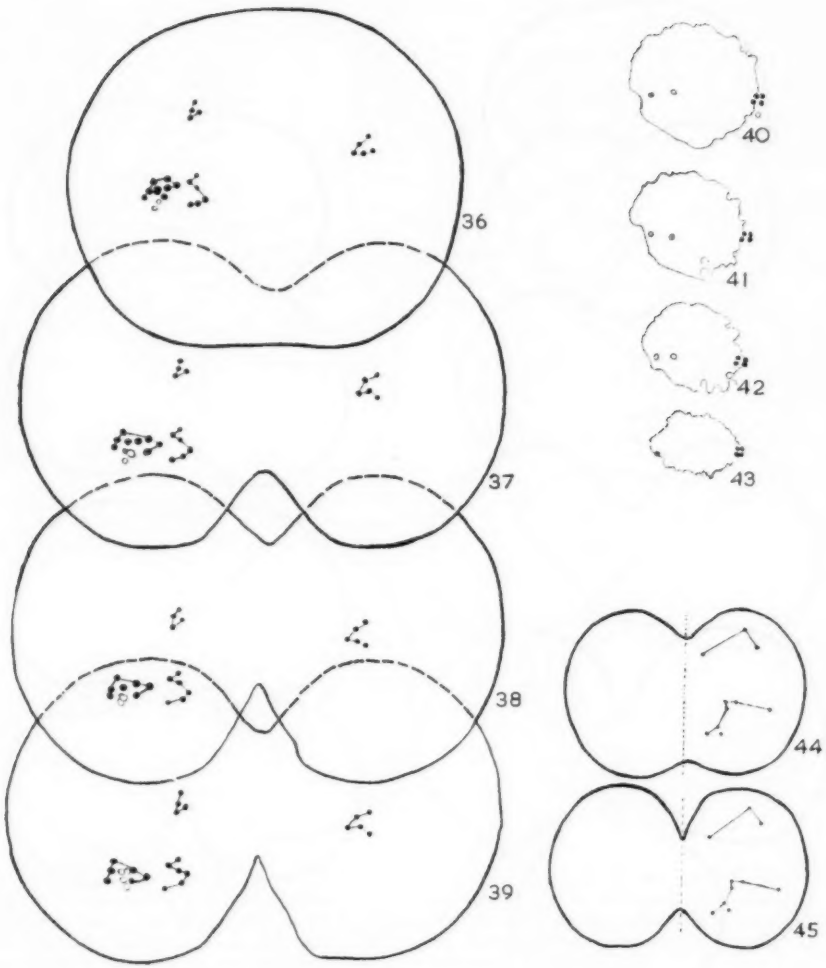
FIGURES 22–28. Granule patterns in the furrow region. FIGURES 22–24. Deformation of granule patterns in the deepening mid-furrow. FIGURES 25–28. View from polar axis, showing iris-like closure of the inner furrow margin of a one-quarter blastomere involving four stages of shrinkage of a pattern of four granules. The relative linear distance between the granules at each stage is illustrated in the numbered diagrams connected by dotted lines.

Such eggs flatten like biscuits as they go off round (Scott, 1946). The flattened surfaces represent regions where the shrinkage is greatest in the initial stage, and vectors of shrinkage occur in more than one direction. They are first marked by concentrations of granules at the animal and vegetal poles. During the formation and the deepening of the multiple furrows, the central island shrinks only in directions tangential to the neck of incipient blastomeres. A triastral cleavage (Figs. 29–33) shows that shrinkage occurs around the blastomere neck so that granules



FIGURES 29–35. Intersecting furrows in polyspermic eggs. FIGURES 29–33. Triastral cleavage. A circumferential shrinkage in the furrows is shown by the movement of granules xxx; the area marked by the triangle does not shrink.

FIGURES 34–35. Tetrastral cleavage. Granule patterns indicate circumferential shrinkage in the dominant furrow and stretching in a direction at right angles.



FIGURES 36–45. Granule patterns on the lateral surface and in the endoplasm. FIGURES 36–39. Lateral surface of the egg, showing the relative immobility of granule patterns. Ishizaka's stationary ring. FIGURES 40–43. Furrow plane and inner furrow margin of an egg in first cleavage; four granules imbedded in the cortex ride on the margin while endoplasmic granules remain motionless. FIGURES 44–45. Endoplasmic granule patterns in a mid-optical section move away from the furrow plane with little distortion.

x-x-x converge but there is a central island, marked by the triangle, which does not shrink, although it might be expected to on the basis of the surface expansion theory (Mitchison, 1952; Swann, 1952). Two stages of a tetrastral cleavage are shown in Figures 34 and 35. In this case one furrow, at the arrow, is in advance of the other and the area of intersection behaves as an integral part of the more advanced furrow. There is marked shrinkage of the surface between the arrows while the "island" surface is, in fact, elongating in the plane of the delayed furrow.

Lateral surface

Ishizaka (1958) has disclosed that two ring areas about the cell can be regarded as stationary if their position during furrowing is related to the center of mass (equator region) and if the spindle axis is fixed. He called them the stationary rings. The position of the rings is determined on two-dimensional outline figures and the term "stationary" refers essentially to the unchanging distance between the ring plane and the equatorial plane on a two-dimensional figure. Now, actually, and Ishizaka realized this, the ring boundaries move further from the equator in a three-dimensional figure when over-the-surface distances are considered. It does not astonish us that the longitudinal expansion of the furrow and of the pole compensate at the latitude of the rings and that they do indeed remain essentially stationary (Figs. 36–39), but while there is little translation of this lateral surface, it does undergo a steady stretching in a longitudinal direction during the whole of the furrowing (see the same Figures 36–39). It results, then, since the lateral surface is relatively fixed, that the subdividing endoplasm within undergoes massive translocation towards one or the other pole.

Endoplasm

A few echinochrome granules remain in the endoplasm and it can be seen that those located in the furrow plane, but outside the spindle, lie quietly in place until the inner furrow margin reaches them (Figs. 40–43). Granules located in a mid-longitudinal plane, away from the furrow, are moved, with some distortion of pattern, further away from the equatorial plane (Figs. 44 and 45). Evidently most of the endoplasm is a deformable plastic mass rather than fluid.

Discussion: Which Theory of Cytokinesis?

Spindle-thrust. In many ways our observations represent a thorough-going confirmation of those made by Dan and his associates. We have substantiated the initial shrinkage of the presumptive furrow. We concur as regards polar, subpolar and subfurrow stretch during furrowing. Yet while we confirm the data, we must reject Dan's spindle-thrust theory. If the 1908 polar abscission experiments of Yatsu on the egg of *Cerebratulus* are not sufficiently conclusive, surely the demonstration that furrowing continues after removal of the amphiaster (Hiramoto, 1956), has made any astral-thrust theory untenable.

Cortical growth. Observations on the newt egg give compelling evidence that a new unpigmented cortex forms in the endoplasm below and ahead of the forming furrow (Selman and Waddington, 1955) and the authors believe that contraction

of the new cortex is a factor in the cell division. It is unlikely that a similar process occurs in the sea urchin egg for the following reasons: (1) all of the equatorial endoplasm is translocated away from the equator as the furrow cuts in; (2) the furrow advances while endoplasm pours through the furrow constriction from one blastomere to the other (in hypotonic calcium-free sea water) (Scott, 1946); (3) cleavage continues smoothly during continual agitation of the furrow endoplasm with a microneedle (Mitchison, 1953).

Surface expansion or furrow contraction. No experiment has yet been designed that will give a clear answer as to whether the active force for cytoplasmic division rests in furrow contraction or in expansion of the non-furrow surface. Our reasons for rejecting the expanding surface theory (Mitchison, 1952; Swann, 1952) are the following: (1) There is now substantial evidence that the furrow cortex has different properties than the non-furrow surface, despite the inability of Mitchison and Swann (1955) to measure a physical difference with their ingenious elastimeter; Beams and King (1937) separated a cleavage-active material from the *Ascaris* egg by ultracentrifugation; Marsland (1950) has demonstrated critical pressure-temperature characteristics of the furrow gel. The furrow cortex has been shown to respond in special ways to hypotonic treatment (Scott, 1946); to dinitrophenol and azide (Kuno-Kojima, 1957). The latter worker has, moreover, isolated fragments of furrow cortex with acid and a detergent. (2) We observed (Scott, 1946) that furrowing may occur in hypotonic sea water with one blastomere increasing in volume while the other is shrinking. This is clearly incompatible with expansion theory. (3) We agree with Marsland and Landau (1954) that surface expansion alone could not provide a vector of force which would direct the surface inward beyond the cylinder stage. (4) The islands noted on trispermic eggs do not undergo continued shrinkage in the way that they would be expected to if active polar expansion were compressing the island from several directions.

In the face of the equivocation still remaining, the contraction theory seems simplest; as Marsland and Landau have noted (1954), it requires no second factor, such as a mechanism for spreading the expansion into the furrow, to explain the "dipping in" after the cylinder stage. What we consider to be strong evidence for the contraction theory was presented in our 1946 paper. It was noted that long stalks develop between blastomeres in hypotonic solutions which, although tenuous, are rigid enough to push the blastomeres 18 micra apart while they continually diminish in diameter.

We can visualize the active furrow surface as a band of specialized cortex about 15 micra wide on the uncleaved egg. We recognize it as different from the rest of the cortex by virtue of the fact that it shrinks, at least in two dimensions, in the initial stage of furrowing. This band of special cortex shrinks most in the center of the furrow and less so on each side, merging into a region which never shrinks at all in the longitudinal direction. Furrow surface shrinkage, which is two-dimensional until the cylinder stage, is circumferential only, around planes parallel to the equator, from the cylinder stage on. The question arises as to why the furrow cortex, previously capable of two-dimensional contraction, is now limited to circumferential contraction around the equator. One might assume that the potentiality for two-dimensional contraction still exists and could

assert itself at any time that resistance could be overcome, but the now cylindrical form and the semi-solid structure allow only the circumfurrow vector of contraction to operate while the longitudinal vector is overcome and the furrow surface expands in the longitudinal direction. In this way we are able to explain the oriented contraction without assuming a pre-orientation of contractable micelles around the equator.

We consider that our observations are in substantial agreement with the contraction hypothesis as formulated by Marsland and Landau (1954). In one detail, however, we can not support their theoretical views. According to their hypothesis, the cortical gel at the furrow peak undergoes solation, with the result that subfurow cortex moves into position at the furrow peak. The authors put it as follows (Marsland and Landau, 1954, p. 532): "Thus more and more cortical gel, possessing an unexpended fund of contractile energy is brought into an operative position as the furrow deepens. Therefore, in the final stages of furrowing, to complete the cleavage it is only necessary to assume that the region of active contraction shifts from the trough of the furrow to the side-walls, first to the region immediately adjacent to the trough and later, to a more peripheral site, somewhat removed from the trough (Stages 4 and 5). In this way, the gel at the very bottom of the trough, having performed its contractile function, could undergo solation, clearing the way for the approach and final fusion of the cell membrane, which severs the stalk between the daughter cells." We have observed, however, that the echinochrome granules are scattered throughout the thickness of the cortex; we have seen no evidence of a non-granular under-layer, which, if present, might undergo solation. Furthermore, as far as we can tell, no furrow granules are liberated to the endoplasm during division—either at the furrow peak or elsewhere. What one observes in fact, is a steady, circumferential contraction of the furrow cortex from the beginning to the end of the process; the cortex changes its shape exceedingly but maintains its integrity throughout. It is possible that new material possessing contractile capacity is added *among* the granules of the furrow, and that the material whose contractile power has been exhausted is expelled, but the basic fabric of the furrow cortex is not destroyed and in any case no granules escape to the endoplasm.

Summary

1. A study of cell surface changes during cell division has been made, using echinochrome granules as natural markers rather than adherent bits of clay or dye.

2. The polar surface of the sea urchin egg stretches symmetrically in all radii from the pole during the first two-thirds of furrowing, and either remains at maximum stretch or shrinks somewhat during the latter third.

3. The furrow surface shrinks in two dimensions in the plane of the surface during earliest furrowing to the cylinder stage; it shrinks latitudinally in a degree which is proportional to the distance from the equator and it expands longitudinally from the cylinder stage on; the cortex undergoes plastic deformation at the peak of the furrow; there is no liberation of cortical echinochrome granules to the endoplasm at any stage of division.

4. All of the cortex is relatively rigid throughout division. The first signs of softening occur at the poles during late furrowing.

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MECHANISMS OF REMOVAL OF INJECTED MICROORGANISMS FROM THE AMERICAN OYSTER, CRASSOSTREA VIRGINICA (GMELIN)[1, 2, 3]

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Extensive studies on the amoebocytes[4] of oysters have elucidated their importance in digestion, but these cells have not been extensively studied as regards their role in defense. It is clear that in this filter-feeding mollusc, amoebocytes move freely through the gut epithelium and engulf partially digested particulate food (Yonge, 1926; Takatsuki, 1934). Intracellular digestion follows and the products of digestion are presumably made available to all the cells of the body. In other Mollusca, complex digestive organs are present and intracellular digestion plays a minor role (Yonge, 1937). In such animals amoeboid cells associated with certain tissues and organs act as specialized defensive agents (Metchnikoff, 1901; Cuénot, 1914).

Stauber (1950) demonstrated that carbon particles (India ink) injected intracardially in the American oyster, *Crassostrea virginica,* are taken up by phagocytes and removed from the tissues as these cells migrate through epithelia to the exterior. However, intracardially injected red blood cells are eliminated both by migration of phagocytes and by intracellular digestion (Tripp, 1958). These studies suggest that two separate defense mechanisms are involved in the oyster, and a study of the relative importance of these processes in relation to injected microorganisms serves as the basis for this report.

Materials and Methods

1. Maintenance of oysters (C. virginica)

The oysters used in these experiments were obtained from either Delaware Bay or the Navesink River at Red Bank, New Jersey. They were thoroughly scrubbed with a wire brush to remove encrusting organisms and mud, and then were maintained in groups of eight or fewer in three-gallon Pyrex battery jars containing one gallon of sea water. This water was of the same salinity as that from which the oysters were originally obtained and was changed twice daily. All aquaria were constantly aerated and were maintained at $17 \pm 1^\circ$ C.

[1] Part of a thesis submitted to the graduate faculty of Rutgers, the State University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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[3] This investigation was supported by a research grant (E-781-C) from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health.

[4] The terms amoebocyte, phagocyte and leukocyte are used interchangeably. The cells referred to are found free in the blood and are both amoeboid and phagocytic.

In preparation for intracardial injection a small hole was made (using a coarse wood rasp or a ¼" electric drill) in the left valve of the shell, directly over the heart. Once the nacreous layer had been penetrated, the opening was carefully enlarged with forceps until the entire heart was visible. A scalpel incision through the mantle and pericardial membrane then exposed the heart. These wounds began to heal within a few days and many oysters formed new shell before they were sacrificed.

Preliminary experiments demonstrated that the adductor muscle was also suitable for injection. The muscle bundles are bathed by extensive blood channels which drain directly into systemic vessels so that material injected into the muscle very quickly reaches the systemic circulation. In preparation for intramuscular injections both valves of the shell dorsal to the adductor muscle were filed so as to expose the muscle, caution being taken to avoid injury to the subjacent tissue.

Prior to injection each oyster was appraised as to its general physiologic condition in terms of strength and regularity of heart beat, frequency of shell closure, and ejection of fecal strings. Animals which did not appear normal were discarded.

2. Maintenance of microorganisms

All microorganisms selected for injection could be recognized by colonial characteristics in agar pour plates and, thus, could be distinguished from the endogenous bacterial flora of oysters.

A strain of *Bacillus cereus* var. *mycoides* (*B. mycoides*) was obtained from the Institute of Microbiology, Rutgers, the State University. Cultures of *Staphylococcus aureus, Escherichia coli, Pseudomonas fluorescens* and *Saccharomyces cerevisiae* were obtained from the Department of Bacteriology, Rutgers, the State University. *Flavobacterium invisibile* was isolated from oyster heart blood. Microorganisms were subcultured routinely on nutrient agar slants, but for purposes of preparing inocula, organisms were grown as outlined in Table I.

All cultures in liquid media were harvested by centrifugation and washed three times with 15 ml. of sterile 0.85% saline. Spores were harvested from agar plates

TABLE I

Media, incubation times and temperatures for various microorganisms used in injecting oysters

Organism	Medium	Incubation	
		Temp. (°C.)	Time (Days)
Bacillus cereus var. *mycoides*		25	5
spores	Nutrient agar (dist.)*	then 5	7
vegetative cells	Nutrient broth (dist.)	25	3
Staphylococcus aureus	Nutrient broth (dist.)	37	1
Escherichia coli	Nutrient broth (dist.)	25	1
Pseudomonas fluorescens	Nutrient broth (dist.)	25	1
Flavobacterium invisibile	Nutrient broth (sea)	25	1
Saccharomyces cerevisiae	Sabouraud broth (dist.)	25	2

* Difco Bacto-nutrient agar, broth or Sabouraud broth rehydrated with distilled or sea water.

and were similarly washed. Final suspensions of organisms were prepared in autoclaved sea water of the same salinity as that in which the oysters were maintained. Prior to injection, the concentration of microorganisms in each inoculum was estimated by dilution plate counts.

3. Injection procedures

Spores and vegetative cells of *B. mycoides*, and vegetative cells of *Staph. aureus*, *F. invisibile* and *S. cerevisiae* were injected intracardially. Vegetative cells of *E. coli* and *P. fluorescens* were injected into the adductor muscle.

All injections were done with a 1.0-ml. tuberculin syringe fitted with a 27-gauge (¾″) or a 30-gauge (1½″) needle. Each oyster was placed in sea water and observed with a binocular dissecting microscope while the needle was inserted directly into the ventricle or adductor muscle; 0.2 ml. of inoculum was injected over a period of several seconds. Oysters received 10^5–10^9 microorganisms, depending on the species of organism and the nature of the experiment. Withdrawal of the needle allowed the wound to close and little of the inoculum was lost. Animals from which excessive leakage occurred were discarded. Each inoculated oyster was passed through six changes of fresh sea water, during which the area over the injection site was flushed gently with a pipette to remove extraneous microorganisms. The oysters were then returned to aquaria and maintained as described above.

4. Sampling procedures

Two types of experiments were performed in order to determine the fate of injected microorganisms. In one group of experiments oysters which had received intracardial injections of *B. mycoides* or *S. cerevisiae* were relaxed, fixed, and prepared sections were examined microscopically. In the second group of experiments tissues from experimental oysters were cultured (at intervals) to recover microorganisms.

a. Sections of inoculated oysters

Preliminary experiments showed that tissue shrinkage due to fixation and dehydration could be minimized by relaxing intact oysters in 0.4 *M* magnesium sulfate at 4° C. for several hours prior to fixation of the whole oyster in Bouin's solution overnight. Ten oysters were injected intracardially with a suspension of *B. mycoides* and one animal was sacrified at each of the following intervals after injection: 1 hour, and 1, 2, 4, 5, 8, 9, 12, 16 and 20 days. Similarly six oysters were injected intracardially with a suspension of *S. cerevisiae* and one animal was sacrificed at each of the following intervals thereafter: 1 hour, and 1, 2, 3, 4 and 12 days. Two or three control oysters were injected with equal volumes of sterile sea water and sacrificed at suitable intervals in each experiment. Examination of control animals disclosed no histological abnormalities or microorganisms.

Following initial fixation the soft parts were removed from the shell, divided transversely into six approximately equal parts and placed in fresh Bouin's solution for 1–4 hours. These blocks of tissue were then washed, dehydrated, cleared,

imbedded in paraffin, sectioned at 10 μ and stained with Gram's tissue stain. Two sections were taken from each block, one representing the anterior edge of the tissue and one from the center of the tissue. Thus, for each animal 12 transverse sections were obtained at regular intervals along the anteroposterior axis.

Certain vessels, sinuses and tissues were regularly examined for microorganisms under high dry and oil immersion objectives. These included: heart, anterior and posterior aortae, large arteries of the visceral mass, subepithelial blood sinuses of the gut, sinuses between digestive diverticula, dorsal and ventral circumpallial arteries, proximal mantle vessels, medial and lateral gill axis sinuses, vertical gill vessels, palp arteries and the blood spaces of the adductor muscle and kidney. In addition, the following epithelial areas were examined for microorganisms: walls of the alimentary canal at all levels, walls of twenty of the digestive diverticula, inner and outer aspects of the palps, outer aspect of the mantle, nephridial tubules, gonaducts, epicardium and pericardium.

b. Cultivation of microorganisms from tissues

Groups of 20–25 oysters received injections of microorganisms suspended in sterile sea water, while 6–8 controls were injected with sterile sea water only. Estimates of the numbers of microorganisms in heart blood, mantle, visceral mass and in aliquots of homogenized whole oysters were made at intervals of one hour, one day, two days, and irregularly thereafter for 6–50 days.

At each interval three experimental and one control oysters were tested for microorganisms according to the following scheme. Each heart yielded 0.1–0.5 ml. of blood which was added to 4.5 ml. of sterile distilled water. Portions of tissue, approximately 0.1 ml. in volume, were removed from the mantle and visceral mass and ground separately in Ten Broeck tissue grinders containing 5.0 ml. of sterile distilled water. The remaining soft parts were homogenized in a Waring Blendor containing 20 ml. of sterile distilled water, and the actual volume of the soft parts was determined by displacement. One-tenth ml. of the material from each tissue or homogenate sampled was placed in each of three Petri dishes, melted agar added and the plates incubated at the optimum temperature for the species

TABLE II

Media, incubation times and temperatures for bacteriologic tests of oyster tissues

Organism	Medium	Incubation	
		Temp. (°C.)	Time (Hours)
Bacillus cereus var. *mycoides*			
spores	Nutrient agar (dist.)*	30	24
vegetative cells	Nutrient agar (dist.)	30	24
Staphylococcus aureus	Nutrient agar (dist.)	37	24
Escherichia coli	Nutrient agar (dist.)	37	24
Pseudomonas fluorescens	Nutrient agar (sea)	25	48–72
Flavobacterium invisibile	Nutrient agar (sea)	30	18
Saccharomyces cerevisiae	Sabouraud agar (dist.)	30	18–24

* Difco Bacto-nutrient agar or Sabouraud agar rehydrated with distilled or sea water.

of microorganism being studied. Individual colonies were counted, averaged and the number of viable microorganisms per ml. of sample was calculated. Using these data, and estimates of the volumes of mantle and visceral mass (respectively 30% and 40% of the soft parts) derived from previous experiments, it was possible to estimate the total number of viable microorganisms in these organs. Results are expressed as the mean values from three oysters. In general, values obtained were in close agreement and only rarely were extreme variations noted. Since there was no suitable method of estimating the total volume of oyster blood, the number of microorganisms recovered from blood samples is expressed per ml. of heart blood sampled.

Results

1. Observations based on tissue sections following injection of yeast cells

Masses of yeast cells seemed to occlude main arteries immediately after intracardial injection. Phagocytosis began immediately and by 24 hours phagocytes contained approximately 95% of yeast cells visible in sampled areas of tissue sections. Tissue sections from the oyster fixed at 24 hours showed that most leuko-

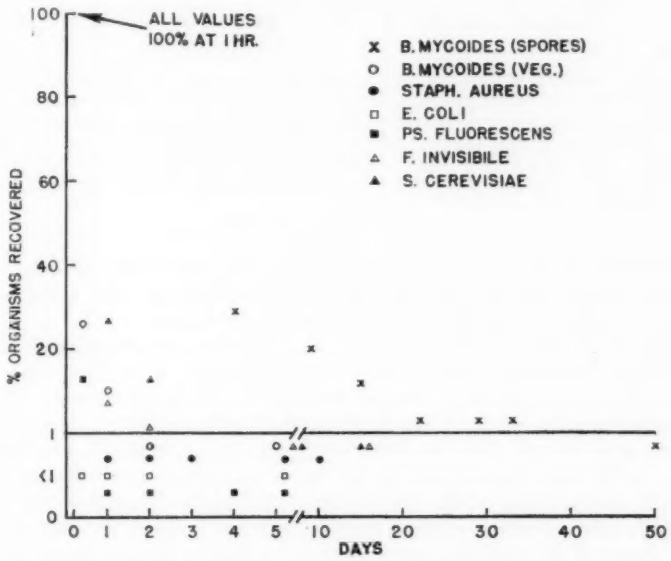

Figure 1. Survival of injected microorganisms per ml. sample of oyster heart blood taken at intervals up to 50 days. Base line samples at one hour represent 100% recovery, and subsequent results are expressed proportionately. Each point represents the mean number of microorganisms from three oysters sampled. Points in the area labeled "< 1" are not quantitative measurements, but indicate that small numbers of bacteria were detected for several days following injection. The last point shown for any one microbial species indicates the termination of a sampling series.

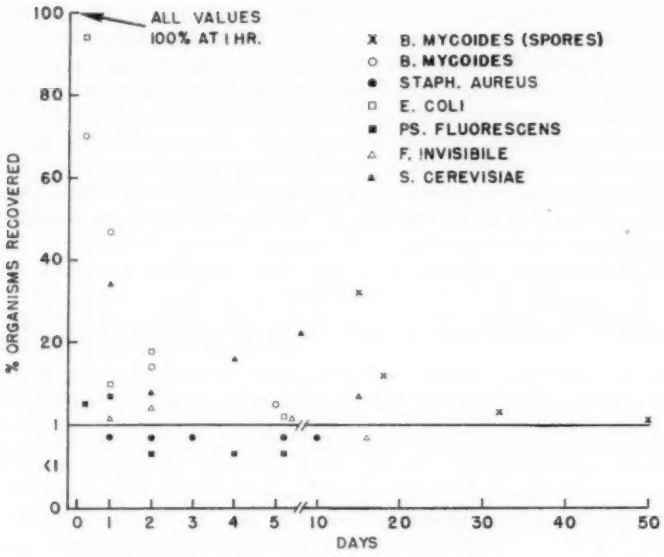

FIGURE 2. Survival of injected microorganisms in samples of oyster mantle tissue at intervals up to 50 days. Base line samples at one hour represent 100% recovery, and subsequent results are expressed proportionately. Each point represents the mean number of microorganisms from three oysters sampled. Points in the area labeled "< 1" are not quantitative measurements, but indicate that small numbers of bacteria were detected for several days following injection. The last point shown for any one microbial species indicates the termination of a sampling series.

cytes contained 2–4 yeast cells, some 8–10 yeast cells, and a few as many as 15–20 yeast cells. It was also evident that appreciable numbers of yeast-laden leukocytes had begun to migrate from blood vessels into adjacent tissues.

During the second day phagocytosis and migration progressed with the result that although individual yeast cells were still present in smaller vessels, large masses of yeast cells were no longer present in the main vessels. Phagocytes containing yeast cells were first seen in the epithelium lining the gut and the digestive diverticula at 48 hours. By the third and fourth day a relatively massive investiture of yeast-bearing phagocytes was evident in the epithelium of the gut, digestive diverticula, palp and mantle, but rarely were such leukocytes found in the epithelium of the gills, kidney, pericardium or gonaducts. Few yeast cells remained in any of these tissues at 12 days post-injection and it appeared that the majority of yeast cells had been eliminated by the migration of yeast-bearing leukocytes to the exterior.

Substantial variations observed in the morphological and tinctorial properties of a small proportion of yeast cells were thought to represent the effects of intracellular digestion. The normally oval or circular outline of some yeast cells became irregular as early as the fourth day post-injection, and instead of appearing a uni-

formly deep blue these yeast cells were mottled or very pale blue. Ultimately, only faintly staining globules, thought to represent the remains of yeast cells, were seen intracellularly. Morphologically-altered yeast cells were rarely seen outside of phagocytes.

2. Observations based on tissue sections following injection of B. mycoides

Suspensions of *B. mycoides* injected intracardially were composed mainly of short chains of vegetative cells which could be detected in Gram-stained tissue sections up to the ninth day. Examination of heart blood smears and tissue sections taken one hour post-injection clearly showed that large numbers of bacteria had already been phagocytized. Tissue sections from the oysters fixed at one hour and later disclosed that phagocytized bacteria were transported within a few hours from major blood vessels into adjacent tissues, while non-phagocytized bacteria remained in the blood vessels. Within 2–5 days post-injection most of the phagocytized bacteria were removed from the oyster by phagocytes migrating through the gut epithelium, or through the inner or outer surfaces of the mantle.

Phagocytized microorganisms which failed to be eliminated via an epithelial

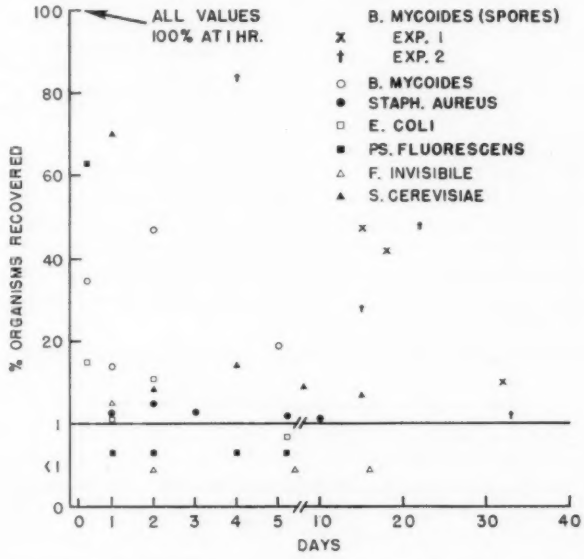

FIGURE 3. Survival of injected microorganisms in oyster visceral mass tissue at intervals up to 33 days. Base line samples at one hour represent 100% recovery, and subsequent results are expressed proportionately. Each point represents the mean number of microorganisms from three oysters sampled. Points in the area labeled "< 1" are not quantitative measurements, but indicate that small numbers of certain bacteria were detected for several days following injection. The last point shown for any one microbial species indicates the termination of a sampling series.

surface underwent vacuolization and loss of Gram-positive character, suggesting that they were dead or dying. Similar changes occurred among extracellular organisms during the first 48 hours after injection. Such degenerative changes were observed only very rarely in control (sea water) suspensions of bacteria maintained at room temperature for similar periods of time.

Except for local accumulations of leukocytes, the significance of which was difficult to evaluate, no distinct pathology was observed in sections of injected oysters.

3. Results of culturing living microorganisms from tissues

Recovery of viable microorganisms from samples of blood, organs and homogenized whole oysters resulted in two distinct groupings of data, one for bacterial spores and the other for vegetative bacteria or yeast (Figs. 1–4). While only a relatively small proportion of injected vegetative cells could be recovered and only within 24–48 hours after injection, bacteria injected as spores were recovered in relatively larger proportions over a period of several weeks. Although the inocu-

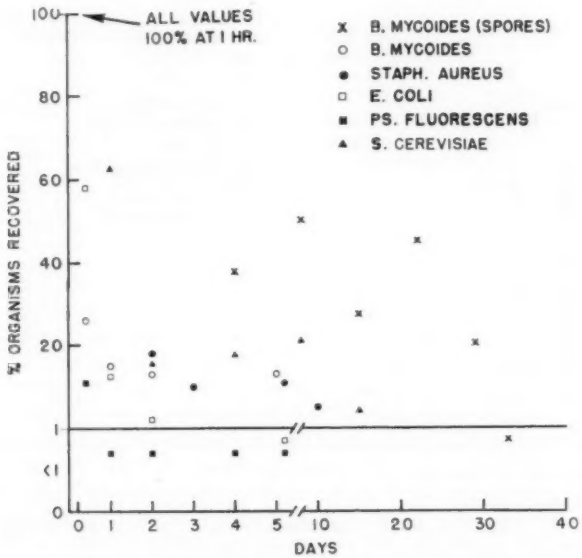

FIGURE 4. Survival of injected microorganisms in whole oysters homogenized at intervals up to 33 days. Base line samples at one hour are assumed to represent 100% recovery, and subsequent results are expressed proportionately. Each point represents the mean number of microorganisms of three oysters sampled. Points in the area labeled "< 1" are not quantitative measurements, but indicate that small numbers of certain bacteria were detected for several days following injection. The last point shown for any one microbial species indicates the termination of a sampling series. (Data for *F. invisibile* are omitted because of contamination of samples.)

lated microorganisms were not completely removed, there was no evidence of bacterial multiplication.

Comment

It is evident that injection of any of several foreign particles into the oyster elicits a basic cellular response. Studies reported here indicate that injected yeast cells or bacterial spores were disposed of by the oyster in the same manner as carbon particles (Stauber, 1950). In all of these cases particles became clumped in efferent vessels and large numbers of leukocytes accumulated around them; most particles were engulfed by leukocytes, removed from the circulation and subsequently transported through epithelial layers to the exterior. These processes are initiated immediately after injection, continue until most foreign particles have

Table III

Method of removal	Present study			Tripp '58	Stauber '50
	Bact. spores	Yeast	Veg. bact.	R.B.C.	Carbon
Phagocytosis and migration	++++	++++	+	++	++++
Phagocytosis and digestion	N.O.	+	++++	++++	N.O.
Extracellular destruction	N.O.	N.O.	++	N.O.	N.O.

N.O.—not observed.

been removed and may be supplemented by intracellular and/or extracellular destruction. On the other hand, the oyster's defense against injected vegetative bacteria seems largely to be dependent upon intraphagocytic digestion. It is possible that oysters maintained under more favorable conditions (*i.e.*, in larger volumes of sea water) would eliminate injected microorganisms even more rapidly. It would be of interest to determine the fate of an oyster pathogen under such circumstances.

Table III summarizes qualitatively the relative importance of various processes in the disposal of injected particles.

The author gratefully acknowledges the encouragement and helpful criticism of Dr. Leslie A. Stauber. Oysters were obtained through the courtesy of Dr. H. H. Haskin, New Jersey Oyster Research Laboratory, Rutgers, the State University. This investigation would not have been possible without the technical assistance of Kenneth Phifer and Sung Yen Feng. Dr. Ruth Gordon, Institute of Microbiology, Rutgers, the State University, kindly supplied the strain of *B. mycoides*.

Summary

1. The fate of injected bacterial spores, vegetative bacteria and yeast cells injected into the oyster, *Crassostrea virginica*, was followed histologically and by viability studies.

2. Tissue sections showed that yeast cells were phagocytized in blood vessels by leukocytes which migrate into surrounding tissues and later through epithelial layers to the exterior. A small proportion of yeast cells remaining in the tissues are digested intracellularly.

3. Histologic examination of oysters injected with living bacteria revealed that the microorganisms were rapidly destroyed both intracellularly and extracellularly before significant numbers of host phagocytes could migrate to the exterior.

4. Viability experiments demonstrated that when several species of non-spore-forming bacteria were injected, very few of these organisms could be recovered 48 hours later. However, a small proportion of these bacteria persisted in oyster tissues for several days.

5. Bacterial spores were removed from oyster tissues at a much slower rate than vegetative bacteria.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY 1960

ABSTRACTS OF SEMINAR PAPERS

JULY 5, 1960

Comparative biochemistry of connective tissue ground substance. MARTIN B. MATHEWS.

Structural proteins of the collagen type, along with neutral and acidic polysaccharides, possibly protein-bound, are typical components of connective tissue. Their conjoint distribution among animal species suggests a broad histogenetic pattern in evolution.

Intensive studies of mammalian connective tissues have led to characterization of a number of acid mucopolysaccharides which may be defined as linear chain acidic polymers with a disaccharide repeating unit containing glucosamine or galactosamine and galactose or an uronic acid. One or more ester sulfate groups may also be present. Acidic polysaccharides from invertebrate tissues are of different composition, with the possible exception of the mactins from clams which resemble heparin very closely. However, heparin (and precursors?) differs from the other mammalian acid polysaccharides in having an intracellular location and α-glycosidic bonds.

Preliminary results of a survey of vertebrates show predominantly chondroitin sulfate A isomer in notochord of lamprey and sturgeon, and predominantly chondroitin sulfate C isomer in cartilage of lamprey, shark, sturgeon and salmon. Keratosulfate is a minor component of these cartilages. The distribution from lowest to highest vertebrate forms suggests that the presence of chondroitin sulfate A and/or chondroitin sulfate C may be a systematic characteristic of vertebrate tissues.

Chondroitin sulfate C-protein complexes of shark cartilage were shown to be aggregates of basic molecular units which have a molecular structure and physical parameters nearly identical to the basic molecular units of chondroitin sulfate A-protein complexes of bovine cartilage. The relatively greater stability of the "C" complexes to heat and urea may reflect an adaptation to the high urea content of shark tissues. The remarkable similarity of molecular structure of "C" and "A" complexes indicates a corresponding similarity of physiological functions and suggests a common plan of molecular evolution of chondroitin sulfate-protein complexes, within which minor structural variations may occur.

Physical properties of muscle extracts. LEONARD NELSON AND W. F. H. M. MOMMAERTS.

Brief extraction of skeletal muscle fixed during rest, contraction or fatigue permits the investigation of changes in the configuration or interactions of the structural proteins consequent upon contractile activity. Paired sartorii, dissected from cold-room frogs and equilibrated in Ringer's solution for 30–90 minutes, at 0° C., were mounted on the electrodes of Mommaerts' quick-freezing apparatus. One muscle served as the resting control, while the other received 40 maximal stimuli in 2 seconds, in an atmosphere of cold moist N_2. During the height of contraction of the stimulated muscle, both were instantaneously frozen in isopentane at about $-160°$ C. The muscles were pulverized at liquid N_2 temperature, warmed to $-10°$ C., and extracted with ice-cold Guba-Straub solution. On dilution with four volumes of cold de-ionized H_2O, the resting muscle extract (RME) formed a voluminous flocculent

precipitate which rapidly settled out, while the stimulated muscle extract (SME) invariably became opalescent, forming a fine particulate suspension only after several hours' delay. (However, if, during the preparation the muscles were equilibrated or stimulated at room temperature, the SME resembled the RME, suggesting that metabolic recovery processes may have intervened.) Centrifugation for 10 minutes, at 5000 × g. yielded 2–3 times greater volume of residue in the SME than in the diluted RME, although the total protein (Lowry method) was about 6–8% of the weight of the frozen muscle in both cases. Similar results were obtained from muscles equilibrated in solutions containing 1 mM. iodoacetate and 2 mM. lactate, one of the muscles stimulated to exhaustion at 0° C. (36 tetani). In a preliminary experiment, addition of 0.7 mM. ATP to aliquots of IAA-treated and extracted muscle caused formation of fine particulate precipitates in both resting and exhausted preparations within 10 minutes of dilution. Addition of 1 mM. creatine phosphate to other aliquots of the same preparations resulted in the appearance of large flocs in the RME and a 10-minute fine particulate in the SME. One may consider that after prolonged stimulation at 0° C., a substance such as ATP, or a precursor able to maintain ATP in the extract, may have been depleted below a critical level necessary to stabilize the actin-myosin interaction exemplified in the "super-precipitation" of the diluted resting muscle extract; while, at room temperature, biosynthetic reactions may restore this substance to the critical level.

JULY 11, 1960

A four-factor ionic hypothesis of spike electrogenesis. HARRY GRUNDFEST.

Potassium inactivation in electrically excitable membrane (*Prog. Biophysics,* **7**: 1, 1957) may be produced by alkali earth and onium ions, procaine and other agents, including inward (hyperpolarizing) or outward (depolarizing) currents. The chemical agents may or may not at the same time increase resting membrane resistance. Thus, pathways of resting and active potassium conductance may be pharmacologically different. Pharmacological agents also affect solium conductance mechanisms. Differences in type and degree of action of one agent in different cells, and of various agents in the same cell indicate that the varieties of electrically excitable membrane may be as diverse as are those of the electrically inexcitable (*Ann. N. Y. Acad. Sci.,* **66**: 537, 1957). While in many cells potassium inactivation is caused by inward currents and this gives rise to hyperpolarizing responses, in frog muscle fibers and eel electroplaques inactivation is caused by outward currents and results in anomalous rectification.

Operation of potassium inactivation (j), in addition to sodium activation (m), its inactivation (h) and potassium activation (n), permits explanation of phenomena that appear to be discrepant in the 3-factor theory. These are: conversion of arthropod muscles from graded to all-or-none responsiveness; properties of graded responses in eel electroplaques; the numerous varieties of prolonged spikes; effects of acetylcholine on cardiac spikes; change in K-electrode property; hyperpolarizing responses; anomalous rectification; passive resistance properties in spikes of frog axons and eel electroplaques; initial after-impedance in squid axons; oscillatory and "upside-down" potentials; various types of "stable states" in membrane potentials, and loss of delayed rectification.

JULY 12, 1960

X-ray-induced dissociation of the mitotic and micromere "clocks." RONALD C. RUSTAD.

X-irradiation of sea urchin gametes or zygotes can lead to the formation of micromeres at the first, second, or third cleavage rather than the fourth. Although cell division is delayed, micromeres are formed at the same time as the normal 16-cell stage. High doses, which induce multipolar spindles, usually obscure the effect, but occasionally micromeres are formed at the first multipolar division.

The biological clock which controls the time of micromere formation is independent of division *per se*, and of any event which occurs just once in each mitotic cycle.

The radiation-sensitive event normally occupies one-third of the mitotic cycle. Several workers have shown that the mitotic delay is expressed in this cytological stage. A hypothetical nuclear clock which operated continuously during interphase would lead to early micromere formation after x-rays because of excess interphase time. Similarly, a clock operating during the x-ray-sensitive phase would lead to early micromeres. A nuclear clock which ran during a non-delayed stage would lead to late micromere formation. The synchrony of micromere formation in control and irradiated zygotes suggests that the biological clock which controls the time of micromere formation is localized within the cytoplasm.

The giant cleavage spindle of the egg of Polychoerus carmelensis. DONALD P. COSTELLO.

This small acoel turbellarian is hermaphroditic, and after reciprocal mating, the eggs undergo fertilization and maturation within the body of each worm, advancing to the metaphase of the first cleavage division, where they come to rest with the chromosomes undivided. These eggs are up to 250 to 260 microns in diameter, if fixed at resting metaphase. The amphiastral spindle is a typical metazoan spindle, measuring up to 120 microns between the central bodies and up to 65 microns in diameter at the equatorial plate. All 34 chromosomes are J- or V-shaped, and arranged in a ring at the periphery with mantle fibers attached to the kinetochores. Continuous fibers fill the central spindle area. The centrioles are rods (straight or bent) up to 5 microns in length and one-half micron in diameter. They are usually oriented at right angles to the main axis of the spindle and, if one examines the centrioles at the two ends of any given spindle, at right angles to each other. This suggests that the centrioles of a resting spindle are "out of phase" with each other, and supplements some electron microscope observations of Porter (and of Burgos and Fawcett) indicating that recently divided centrioles in vertebrate material are cylinders at right angles to each other.

E. D. Hanson has recently revived a theory suggesting that the acoel Turbellaria originated from the ciliate Protozoa. If so, he suggests that the acoels should lack centrioles and should possess intranuclear spindles, like the ciliates. Such is obviously not the case, either in the egg, the blastomeres, or in the relatively rare division figures found in the parenchyma of young worms. Von Graff (1891) for *Monoporus rubropunctatus* and Gardiner (1898) for *Polychoerus caudatus* had early figured metazoan-type spindles in acoel eggs.

Aided by a grant from the National Institutes of Health, RG-5328.

Motion pictures of radiation-induced modifications in the early development of Arbacia. CARL CASKEY SPEIDEL AND RALPH HOLT CHENEY.

Graded exposures of gametes to either ultraviolet (2537 Å) or x-ray irradiation, with subsequent fertilization, were effective in bringing about deviations from the normal in developing sea urchins. These deviations included abnormalities in structure and delay in the developmental time schedule. Dosages ranged from very weak to very strong. The extent of radiation-induced injury, the amount of developmental retardation, and the maximal limit of differentiation and growth attained were all directly related to the dosage strength.

Illustrative cinephotomicrographs were obtained of the following features: (1) movements of intracellular constituents of irradiated eggs immediately before and after fertilization, (2) variations in the formation and elevation of fertilization membranes, (3) protoplasmic and granular movements during the first 90 minutes, culminating in normal and abnormal first and second cleavages, (4) aberrations in size and number of blastomeres in later cleavage stages, (5) initiation of twirling and swimming movements of blastulae, and (6) differentiation of gastrulae and plutei.

Time-lapse motion pictures gave a vivid portrayal of the radiation-induced changes visible in the early stages (1–4 above). Conspicuous viscosity changes were apparent in both the superficial hyaline layer and in the deeper protoplasm. After severe radiation the death throes of a zygote or of a later blastomere were frequently accompanied by accelerated internal upheaval. Normal rate pictures were more suitable for depicting the changes in the later stages (5 and 6 above). Crippled progeny included lopsided twirling gastrulae and misshapen plutei with abnormal patterns of arms, skeleton, and other features. There were both significant

resemblances and differences in the various modifications induced by the two kinds of radiations.

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JULY 19, 1960

Electron spin resonance study of serotonin-FMN interaction. IRVIN ISENBERG.

The riboflavin semiquinone at strong acid pH gives a characteristic signal, using electron spin resonance techniques. If serotonin and riboflavin are mixed at pH less than one, a signal is obtained in spin resonance that looks similar to the riboflavin signal but has important differences. The similarity indicates that the riboflavin semiquinone is produced but the differences indicate that another free radical is also produced.

Previous spectrophotometric investigations have indicated that serotonin and riboflavin form a strong charge transfer complex. The present work may be interpreted as indicating that in strong acid solutions, the complex may dissociate into two free radicals, one of which is riboflavin. The other may be a serotonin free radical or some secondary free radical resulting from a reaction of the serotonin free radical.

Contractile responses in the presence of electron donors and acceptors. BENJAMIN KAMINER.

Isenberg and Szent-Györgyi have demonstrated that charge transfer complexes occur between electron donors and acceptors of biological importance. This led to an investigation on the effects of combinations of some electron donors and acceptors on smooth and striated muscle. In the case of a smooth muscle preparation (the proboscis of *Phascolosoma gouldii*) it was previously reported that preparations which did not respond to serotonin (a good electron donor) could be induced to contract by pretreatment with electron acceptors such as riboflavin, acridine red or sevron blue. Further investigations on striated muscle (sartorius of *Rana pipiens*) demonstrated that a good electron donor such as iodide (NaI having been substituted for the NaCl in Ringer's solution) or caffeine (10^{-3} *M*), could induce contracture, provided the muscle was pretreated with an electron acceptor such as iodine ($0.26–1.3 \times 10^{-4}$ *M*) or 1,2,-naphthoquinone (10^{-3} *M*); each substance alone, however, did not produce such an effect. The contracture varied in amplitude and, when marked, was usually irreversible and associated with a loss of response to electrical stimulation. It should also be noted that the iodide and caffeine have another similar action in that they both increase the twitch tension. Thus it appears that in both smooth and striated muscle, contractile responses are obtained with electron donors when in the presence of electron acceptors. These findings suggest the possibility that electron transfer could be involved in excitation of muscle. (This work was done during the tenure of a Rockefeller fellowship.)

Detection of electron donors. ANDREW HEGYELI.

Good electron donors, such as indole, give a strong colored charge transfer complex with iodine, which is a "local" electron acceptor. This phenomenon can be used in paper chromatography for the detection of good electron donors in tissue extracts. In order to localize the substances with good electron-donating properties on the chromatogram, the paper is exposed to iodine vapor. For this purpose we have a glass cylinder which contains, at the bottom, dishes filled with iodine crystals. Over them is a propeller driven electromagnetically. This distributes the iodine vapor uniformly.

JULY 26, 1960

The action of trypsin on acetylmyosin. W. ROBERT MIDDLEBROOK.

Myosin was acetylated by treating it with excess acetic anhydride in 1 *M* K_2HPO_4 at 0°. The resulting material was soluble in water, and stable to freezing and freeze-drying. Ap-

proximately 100% of the epsilon amino groups of lysine were acetylated. The material behaved similarly to myosin in the ultracentrifuge and separated into two peaks after digestion by trypsin, similar to heavy and light meromyosins. The digested acetylmyosin was depolymerized into sub-units with an average chain weight of 5000, on treating for 24 hours in 5 *M* urea. The N- and C-terminal groups were identical with those obtained from the meromyosins. Complete digestion of myosin and acetylmyosin by trypsin was followed in the pH stat. The difference between the asymptotic values of the two curves was equivalent to the lysine content of the myosin, and could be explained by the fact that acetyl-lysyl peptide bonds are not hydrolyzed by trypsin.

The structure of water in tissue, as studied by nuclear magnetic resonance. H. J. C. BERENDSEN.

A useful method for obtaining information about the water in tissue is the study of proton magnetic resonance. When a material containing protons is placed in a strong magnetic field, energy is absorbed in the radio-frequency range, due to the fact that the two possible spin states of the protons have different energies in a magnetic field. The shape of the absorption curve is determined mainly by the interaction and the motion of the protons, strong interactions and slow motion giving rise to broad absorption curves. If, in the study of hydrated macromolecules, oriented samples can be used, additional information is obtained by measuring the absorption as a function of the angle between the sample axis and the magnetic field.

The study of partially dried collagen showed that the classical picture of absorbed water does not hold, but that a considerable structure is present in the water. The best explanation of the experimental data is that in partially dried collagen the water molecules form chains in the fiber direction that rotate restrictively about an axis along the fiber axis. With the natural amount of water present, there is a faster exchange of protons.

A peculiarity of the three-fold collagen helices is that the axially repeating distance along the molecules equals exactly six second-neighbor distances of water, *i.e.*, a-axis repeats of ice (4.74 Å), thus stabilizing the existence of water chains by hydrogen bonds at the right places.

The building of a model showed that water probably can form cylinder-symmetrical structures with a repeating angle of 36° and an axial repeat of 4.74 Å. It is interesting that both deoxyribonucleic acid and collagen have repeating angles of 36° and axial repeats that are multiples of 4.74 Å.

A structural organization of water in tissue has implications for charge and energy transfer and for the theory of sodium extrusion as a bulk property.

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Pinocytosis, phagocytosis, and lysosomes: cytochemical and electron microscopic studies. ALEX B. NOVIKOFF.

Cytochemical stains and electron microscopy permit the study of lysosomes, characterized by: (1) acid phosphatase activity in cell smears or frozen sections (fixed in cold formal-calcium) incubated in Gomori glycerophosphate medium, (2) a single ("unit") outer membrane. In erythrophagocytes, Kupffer cells, lung macrophages, cells of proximal convolution and glomerular epithelium, phagocytosis-pinocytosis vacuoles have been studied in both procedures and have both properties. Acid phosphatase activity is also present in *Chaos chaos* pinocytosis vacuoles, *Ameba proteus* food vacuoles, and vacuoles of spleen macrophages. The distinction between phagocytosis and pinocytosis seems less rigid: the vacuoles are bounded by former cell membrane and appear to have high acid phosphatase (and presumably other acid hydrolase) activities; as light microscopy visualized colloidal particles, electron microscopy now visualizes ferritin molecules in solution. Tracers (ferritin [glomerulus, Farquhar and Palade; *Trypanosoma mega*, Steinert and Novikoff]; colloidal particles [liver, Hampton; proximal convolution, Burgos]; enzymatic proteins—peroxidase, acid and alkaline phosphatase [kidney, liver, Novikoff *et al.*]) indicate that micropinocytosis vacuoles transport materials into larger vacuoles or lysosomes. To determine whether the small vacuoles also are lysosomes will require visualization of low levels of hydrolase activity in well-preserved electron

microscope preparations. Awaiting future study are the origin of acid hydrolases, the relationships of "cytotic" vacuoles and Golgi apparatus, and the quantitative and qualitative significance of "cytosis" in the cell's economy. From their vital dye uptake and acid phosphatase activity (Pasteels, Mulnard, Dalcq), metachromatic granules appear to be lysosomes; from their fine structure (Rebhun) they appear to be "multivesicular bodies" (Sotelo and Porter). These bodies are seen in many cells; their unit vesicles might be formed by micropinocytosis or a membrane-generating process involving the Golgi apparatus.

August 1, 1960

Electrical inexcitability of the frog neuromuscular synapse. Robert Werman.

Frog muscle endplates were explored with an extracellular microelectrode. An intracellular electrode simultaneously monitored invasion into the endplate region of a spike which was directly evoked by a distant microelectrode in the same fiber. In the external recordings from sites generating miniature endplate potentials, an external positivity was invariably seen in association with the rising phase of the intracellular spike. Frequently, a subsequent small prolonged negativity terminated the potential. In contrast, when the exploring microelectrode was moved as little as 5 μ, the external recording became a positive-negative-positive sequence which is characteristic of the approach, local development and onward movement of propagated responses. The negativity was associated with the rising phase of the intracellular spike and its amplitude increased as the exploring electrode was moved further from sites of externally recorded miniature activity. It is generally agreed that the external potential closely follows the time course of the membrane current. Thus, the absence of negativity associated with the rise of the invading spike indicates absence of inward current at sites of externally recorded miniature activity. The late negativity can be accounted for by capacitative current through passive membrane. Since the sites at which the miniature endplate potentials are generated are presumably also the sites of synaptic activation, the synaptic membrane appears not to be excited by an electric stimulus of the magnitude of an action potential.

August 2, 1960

Free radical formation during indophenol blue synthesis by heart muscle respiratory enzymes. Philip Person and Albert Fine.

A study of indophenol blue synthesis from dimethylparaphenylenediamine and alpha-naphthol, as mediated by Keilin and Hartree type beef heart muscle preparations, has revealed that of the above reagents only the diamine is enzymatically oxidized by the heart muscle particulates. Enzymatic oxidation of the naphthol could not be detected using manometric and spectrophotometric methods capable of measuring such oxidations. The first step in the sequence of events leading to indophenol blue synthesis is the oxidation, by the heart muscle particulates, of the diamine to a free radical. The latter is stable and may be identified by its absorption spectrum which contains maxima at 550 and 520 mμ. The free radical forms a dimer, also identifiable by an absorption maximum at 640–650 mμ. Indophenol blue is formed as a result of a non-enzymatic oxidative coupling of the free radical and its dimer with the alpha-naphthol.

August 9, 1960

Cation permeability in muscle. R. A. Sjodin.

If the influx of potassium ions in frog sartorius muscle is determined at various external concentrations of potassium, the results can be fit to the "constant field" equation with a nearly constant permeability coefficient, as long as the system is close to the steady-state. When rubidium or cesium ions are employed, the influxes decline more with rising concentration than predicted by theory. When the potassium content of the muscle is not in the steady-state, the calculated permeability coefficients vary up to 15-fold, depending on the sign and magnitude of the driving force.

In general, the theory that ions in muscle are following simple electrochemical driving forces across the cell membrane appears inadequate and leaves one with the problem of accounting for the variations in measured permeability coefficients. One situation which could provide a basis for the observed departures from the flux equations is the case in which the number of membrane sites for a particular ion is limiting. A collision between an ion and an available membrane site seems a likely prerequisite for membrane entrance. The product of the external concentration and the number of sites available at the outer membrane surface should give a measure of the probability for membrane entrance from the external solution. This probability also involves a factor which represents the interaction energy (affinity) between the particular ion and the site.

It has been possible to demonstrate that the limited site model adequately describes the entry of potassium, rubidium, and cesium ions in frog muscle, either individually or competitively in mixtures. The affinity constant required for each ion remains invarient over a 50-fold range of concentration and flux. The order of decreasing affinity was found to be $Rb > K > Cs$.

Effects of ions on potential in lepidopteran muscle fibers. PETER BELTON.

Nerve-muscle preparations were made from the prothoracic leg of saturniid moths and the longitudinal locomotor muscles of pierid larvae. The effects of changes in external concentrations of K, Na, Ca and Mg on resting and neurally-evoked action potentials (r.p., a.p.) were described. The latter are graded events and are compounded from synaptically and electrically excited components. Changes in r.p. were evoked more readily by variation of K_o than by the other three cations. A KCl solution isosmotic with the control saline did not depolarize muscle fibers immediately but prolonged the falling phase of the synaptic component. Longer soaking produced a neural block or repetitive firing of the muscle as a result of depolarization of the nerve terminals. K-free salines initially shortened the falling phase of the a.p. but later caused a plateau similar to that found in frog muscle with K- and Cl-free salines.

Omission of Na greatly reduced the electrically excitable and synaptic components of the response. When Na was replaced by sucrose, an initial enhancement of r.p. and a.p. was seen.

Seventy mM/l. Mg did not usually delay the take-off of the electrically excitable component, but decreased the size and increased the duration of the a.p. Neither did the omission of Ca block neuromuscular transmission. When responses were evoked at the rate of 0.5/sec. slight increases in r.p. and a.p. were evident in solutions with 30 mM/l. Ca. When paired stimuli were used, the relative refractory period for the electrically excitable component was found to have increased from 10 to about 500 msec. Bursts of stimuli at 50/sec. decreased the size of the responses more rapidly than stimuli at 25/sec. The effect of Ca_o increase was more rapid than that of the other changes described.

It is concluded that other factors can, to some extent, override the K and Na electrode properties of the excitable membrane of these cells.

AUGUST 16, 1960

A scheme for the mechanochemisty of muscle. FRANCIS D. CARLSON.

Our studies on frog muscle have shown that 0.286 μmole/gm. of phosphocreatine are dephosphorylated in an isotonic twitch with a time constant less than thirty seconds. This corresponds to approximately one molecule of phosphocreatine split for each molecule of G-actin in the muscle. Chance and Connelly found only 0.009 μmole/gm. of adenosine diphosphate produced per twitch, and the half time of ADP rephosphorylation was of the order of minutes. The following scheme which reconciles these results assumes, as the stoichiometry cited suggests, that the reversible transformation of G-ATP-actin to F-ADP-actin occurs in the muscular contraction-relaxation cycle. Reaction one: G-ATP-actin is dephosphorylated during the contraction-relaxation cycle to give F-ADP-actin and orthophosphate. Reaction two: F-ADP-actin forms G-ADP-actin during depolymerization. Reaction three: G-ADP-actin is rephosphorylated directly by the creatinephosphokinase system as reported by Strohman, or else indirectly by exchange of traces of free ATP for the bound ADP and subsequent phosphorylation of the latter by the creatinephosphokinase system to give more ATP. These reactions involve nucleotide bound to actin and hence not available to exchange readily with free nucleo-

tide. Reaction four: Phosphocreatine and ADP are formed in the sarcoplasm from the creatine-phosphokinase reaction as a result of the displacement of equilibrium by the creatine produced in reaction three. The equilibrium of reaction four favors creatine and ATP formation; hence only small amounts of ADP and phosphocreatine will be produced. Reaction five: Mitochondrial oxidative phosphorylation, stimulated by the ADP from reaction four, produces ATP which results in the restoration of creatine, phosphocreatine, and ADP concentrations. The slight increase in sarcoplasmic ADP produced by reaction four would correspond to that seen by Chance and Connelly. In the absence of glycolysis and oxidative phosphorylation the creatine and phosphocreatine concentrations would not be restored and our results would obtain.

Reversibility of actin depolymerization in presence of KI. ANDREW G. SZENT-GYORGYI AND EVA M. SZENTKIRALYI.

In presence of 1 mM $MgCl_2$ and 0.1 mM ATP or phosphocreatine (PC) a relatively high concentration of KI (0.8 *M*) is required for the complete depolymerization of purified F-actin. At lower KI concentrations the viscosity of actin has intermediate values. Depolymerization performed in presence of Mg and phosphagens can always be reversed by reducing the ionic strength. If Mg or the phosphagens are omitted, depolymerization is complete already at 0.3 *M* KI and is irreversible. The effect of PC and ATP can be explained by assuming the simultaneous polymerization and depolymerization of actin, a process similar to the findings of Oosawa *et al.* (*J. Polymer Sci.* 37: 1323, 1959) in low concentrations of $MgCl_2$. In accordance with such a picture, the steady formation of ADP, exceeding the stoichiometric ratio, can be measured at the intermediate KI concentrations, but not at 0.8 *M* KI where depolymerization is complete, or at 0.1 *M* KI where depolymerization is very slow. The maximum rate of ADP formation at room temperature occurs at 0.4 to 0.5 *M* KI, and 1 mole of ADP is formed in about 100 minutes by 57,000 grams of actin. The role of Mg in these reactions is specific and cannot be replaced by Ca, neither do ATP and PC act without Mg. During depolymerization G_{ADP}-actin is formed which requires Mg for its stability, possibly by facilitating the binding of the nucleotide to actin. When F-actin is depolymerized in the presence of Mg only, the resulting G_{ADP}-actin will not polymerize when the ionic strength is reduced, even though it is not denatured. Polymerization can be induced, though, by the addition of ATP or PC. The polymerization process thus can be separated into at least two steps. In the first step ATP or PC alters actin in such a fashion that it can polymerize, provided the ionic strength is favorable for the polymerization proper. During the process ATP is dephosphorylated. The nature of the intermediate is not known.

Studies on actin. II. Polymerization and the bound nucleotide. TERU HAYASHI AND RAJA ROSENBLUTH.

The necessity of actin for actomyosin contraction has been established, and the possible role of actin in muscle contraction as a localized, fixed site of phosphate turnover has been revived by Strohman and, more recently, Carlson. A crucial point in these speculations centers on the question—is the actin-associated nucleotide amenable to enzymatic attack while bound to the actin protein? The answer was sought in a study of the properties of G-ADP actin, since theoretically it was the form most likely to be the focal point for phosphate turnover.

At 1° C., G-ADP actin loses ca. 20% of its bound nucleotide in an hour, considerably faster at 29°. This loss is strongly inhibited by 1 mM Mg^{++}. It was found, surprisingly, that G-ADP actin is capable of polymerization upon the addition of salt; that is, it is not necessary to convert G-ADP to G-ATP for polymerization. However, at 1° C., G-ADP polymerizes slowly, whereas G-ATP polymerizes rapidly. In all cases, the extent of polymerization is directly proportional to the bound nucleotide. Thus, the facts have been established for the test of the principal question.

G-ADP actin in 1 mM Mg^{++} (where only traces of bound nucleotide are lost) is incubated with CP and CPase. The resulting solution is tested for polymerizability at 1° C. If the actin polymerizes slowly, it is still G-ADP, and no enzymatic transphosphorylation has occurred. If it polymerizes rapidly, it is G-ATP, and the bound nucleotide has been transphosphorylated. The latter is found to be the case, and it is concluded that the bound nucleotide of G-ADP is capable of enzymatic transphosphorylation.

AUGUST 23, 1960

The three hearts of the oyster. PAUL S. GALTSOFF.

The open circulatory system of the oysters and other bivalves is characterized by the presence of large sinuses and open lacunae or spaces which receive blood from the arteries. It is obvious that under such a condition effective circulation is impossible. This deficiency is compensated by several mechanisms: the pulsation of the radial vessels of the mantle; the squeezing of blood in the vessels of the gill lamellae by the contraction of gill muscles; the contraction of the wall of the common afferent vein; and by the contractions of the two accessory hearts located on the wall of the cloaca on each side of the adductor muscle. The accessory heart is not a simple tubular structure, as it had been heretofore described; it consists of three large branches, joined at a center in the form of a letter Y. Injection of dyes shows that the accessory hearts are in communication with the anterior pallial arteries, the renal sinus, the efferent veins and the circumpallial artery. It is interesting that the blood from the accessory heart enters this vessel against the ventricular pressure of the principal heart. The contraction wave starts from the center of the accessory heart and goes into two opposite directions. The rate of contraction is independent of the beating of the principal heart and is much slower. The function of the accessory hearts is to oscillate the blood within the pallium.

GENERAL SCIENTIFIC MEETINGS

AUGUST 29-31, SEPTEMBER 1, 1960

Abstracts in this section (including those of Lalor Fellowship Reports) are arranged *alphabetically by authors* under the headings "Papers Read," "Papers Read by Title," and "Lalor Fellowship Reports." Author and subject references will also be found in the regular volume index.

PAPERS READ

The pupillary mechanism in the toad. PHILIP B. ARMSTRONG.

The isolated sphincter pupillae of the toad (*Bufo marinus*) can be stimulated to contract by the direct action of light. If maximal light constriction is produced and the illumination of the pupil continued, there is a slight adaptation to the light, as manifested by a slow continuing dilatation of the pupil. The constriction of the pupil produced by light is not antagonized by atropine even in high concentrations (10^{-3}). The threshold for acetylcholine is 10^{-9} with the dilated dark-adapted pupil. Acetylcholine 10^{-5} produces constriction about equal to that produced by maximally effective light. A further increase in the acetylcholine concentration (10^{-4}) results in some dilatation of the pupil but the sensitivity of the constricter to light appears to be unchanged. Acetylcholine is antagonized by atropine. Pilocarpine produced a slight constriction of the pupil but only in high concentrations, the threshold being 10^{-4}. Serotonin produces some slight pupillary constriction also only at high concentrations (10^{-3}). DL arterenol produces a striking dilatation in both the light-adapted and dark-adapted pupils. The threshold is 10^{-9} for the dark-adapted pupil.

Stimulation of the sympathetic trunk at the level of the most anterior vertebrae produces a striking pupillary dilatation, as does also the stimulation of a pair of nerves entering the eyeball at its posterior inferior aspect. It may be that this is a mixed nerve since occasionally a very slight constriction of the pupil can be induced by stimulation of these same nerves when the preparation is dark-adapted.

It appears that the sphincter pupillae has little if any motor innervation, that the dilator is sympathetically innervated probably by adrenergic fibers.

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A method for obtaining gametes from Asterias forbesi. A. B. Chaet and R. S. Musick, Jr.

A new method of obtaining starfish gametes has been developed, enabling one to obtain substantial quantities of fertilizable eggs (and sperm) even during the latter part of August. Animals were injected with an extract prepared from isolated radial nerves of large unripe starfish. The nerves, after washing in sea water, were lysed in distilled water for 10 minutes (1 cc. per nerve), and the supernatant was dialyzed against running sea water for 12 hours (or at 6° C. with frequent changes of sea water). A second treatment with distilled water yielded another fraction which also contained substantial concentrations of "shedding substance." Fifteen-hundredths cc. per gram of the heat-stable extract(s), which may be stored frozen, was injected through an arm into the coelomic cavity of a small, 10–30-gram (about 3-inch) ripe starfish. The animals were kept in dry iced fingerbowls (approximately 17° C.) until shedding was initiated, usually within 45 minutes. The eggs were collected in beakers by suspending the shedding females (oral side up) over beakers of sea water. Fifty to 70% of the animals injected in this manner shed, depending upon the state of the gonads. The eggs of 82% of the females shedding after an injection of the "shedding substance" exhibited 90% or better germinal vesicle breakdown. Preliminary evidence indicated that the eggs were highly fertilizable (88% or above). Yields of as much as 3–5 cc. of eggs were obtained from one 20–40-gram starfish. These small ripe starfish were obtained from cold water and were available until at least the latter part of August. Since the stock animals were stored in refrigerated sea water tanks, shedding could be induced when required. Further studies are being carried out on the characteristics and physiologic function of the shedding substance obtained from the starfish nerve.

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The modification of life span by x-rays for haploids and diploids of the wasp, Habrobracon sp. Arnold M. Clark.

Haploid males, diploid males and diploid females of the wasp, *Habrobracon* sp. (an Indian species related to *Habrobracon juglandis*), show a decrease in adult life-span following exposure to x-rays as larvae-in-cocoons, white pupae or adults. The median life span for non-irradiated adults was 62 days for haploid males and diploid males fed on honey-water, 92 days for diploid females fed on honey-water and 40 days for diploid females fed on larvae of the Mediterranean flour moth, *Ephestia*. This difference in life-span related to sex, but not to genome number, indicates that the aging process is not due to an accumulation of somatic mutations during adult life. Haploid males exposed as adults to 10,000–50,000 r have a shorter life span than comparable diploid males. Diploid males and diploid females show similar decreases in life span relative to their controls. Pupae after exposure to 10,000 and 15,000 r and larvae after exposure to 2,000 r are equal to controls in post-embryonic survival and in ability to develop into structurally normal adults. These adults, however, show a decrease in life-span. Diploid males resulting from irradiated larvae or pupae show a decrease in life-span that is smaller than comparable haploid males but similar to diploid females. Radiation-induced decrease in life-span is markedly influenced by genome number but not by sex. This indicates that in contrast to the normal aging process, the decrease in life-span by x-rays is due to damage to the genetic material.

Distribution and substrate specificity of sea urchin egg phosphatases. Gilles Cousineau and Paul R. Gross.

Phosphoesterase activities of sea urchin egg homogenates have been assayed against a variety of substrates and at three pH values. The substrates employed were glucose-6-phosphate (G6P), α-glycerophosphate (GP), nitrophenyl phosphate (NPP), fructose-1,6-diphosphate (F1,6P), and glucose-1-phosphate (G1P). These substrates were incubated with the enzyme sources at pH's of 5.4, 6.4, and 8.4. Fractions were obtained by differential centrifugation of the homogenates, which were prepared in isotonic KCl from unfertilized eggs that had been

washed, successively, in filtered sea water, Ca-Mg-free artificial sea water, isotonic sodium citrate, and isotonic KCl. The homogenization medium contained 10^{-3} *M* EDTA. The expected activities for acid and alkaline phosphatases were observed. At pH 5.4, setting the hydrolysis rate for glucose-6-phosphate at 100, phosphate release rates for the other substrates were computed as: Fl,6P, 132; NPP, 119; GP, 47; GlP, zero. At pH 8.4, with G6P again set at 100, release rates for the other phosphate esters were: Fl,6P, 97; GP, 47; NPP and GlP, zero. At pH 6.4, where the activities of the acid and alkaline phosphatases could be expected to be minimized, significantly high hydrolysis rates were obtained for two substrates, G6P and NPP. The rate pattern was as follows: NPP, 415; G6P, 100; GP, 17; Fl,6P, 17; GlP, zero. Although the behavior of these systems toward NPP at pH 6.4 constitutes a difficulty, the patterns of activity of the preparations are consistent with the existence, in the sea urchin egg, of a distinct glucose-6-phosphatase, possessing substrate specificities similar to those of the enzyme obtained from mammalian tissues. The homogenate fractions (pigment vacuoles, yolk particles, mitochondria, "microsomes," and soluble phase) showed different but characteristic patterns of activity against the substrates at the different pH values. The essential conclusions of this part of the study are as follows: the distribution of phosphatase activity among the fractions is different at the three pH values chosen, supporting the possibility that the catalysts are distinct from one another. Most of the glucose-6-phosphatase activity, so characteristically a "microsomal" function in mammalian cells, is localized in particles identical with, or sedimenting along with, the yolk granules.

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Osmolarities of some body fluids in the elasmobranch and teleost. H. DAVSON AND CYNTHIA THOMAS GRANT.

Earlier work has shown that, in the mammal, the cerebrospinal fluid (CSF) is hyperosmolar to the blood plasma whilst the aqueous humour is approximately iso-osmolar; in the dog-fish (*Mustelus canis*) the aqueous humour is markedly hypo-osmolar to the plasma. In the present study on the dog-fish the osmolarities of the plasma, aqueous humour, CSF and the "subdural fluid" lying immediately below the cartilage of the skull were found to be 970, 947, 964 and 944 milliosmoles per liter, respectively. Thus, the CSF is approximately iso-osmolar with plasma whilst the "subdural fluid" is similar to aqueous humour. The chloride concentrations followed the same pattern, being 275, 245, 282 and 273 millimoles/kg. H_2O, respectively. In the scup (*Stenotomus versicolor*) the osmolarities of plasma and eye-fluids (a mixture of aqueous and vitreous humours) were 397 and 364 milliosmoles per liter: thus there is a high difference of osmolarity across the cornea of the teleost, but in spite of this the eye-fluids remain hypo-osmolar to plasma. It is likely that the "spectacle" represents an adaptation that slows down the osmotic withdrawal of water from the eye-fluids since removal of this skin from one eye caused a progressive increase in the osmolarity of its eye-fluids; thus, 1, 4, 6 and 10 hours after removal the differences between the two eyes were 10, 17, 31 and 40 milliosmoles per liter. The chloride concentrations followed the same pattern. In one fish it was found that its spectacle was opaque, presumably through damage to its epithelium, and it is interesting that the fluids of this eye were stronger than those of the other by 130 milliosmoles per liter. In *Tautoga onitis* plasma and eye-fluids contained 360 and 321 milliosmoles per liter, respectively. The intraocular pressure of dog-fish and scup was 10 cm. H_2O.

This investigation was carried out in part under a Fight for Sight Fellowship of the National Council to Combat Blindness, Inc., New York.

The relationship between oxygen tension and light production before and after grinding up the light organs of fireflies. ARTHUR B. DUBOIS.

Fireflies of the local variety of Photinus and Photuris, when studied *in vivo*, require approximately 100% O_2 to achieve a maximal steady glow. Extracts of their light organs are said to require only 1% O_2 for a maximal steady glow. This difference may be due to a lower rate of reaction *in vitro*, requiring less oxygen, or to a diffusion gradient for O_2 *in vivo*, yielding a low O_2 tension at the site of reaction. The present experiment was an attempt to estimate the intensity of glow at different O_2 tensions before and after grinding up the light

organ. A photomultiplier system was used to measure the light emission. A small glass tissue grinder was fitted with an air-tight rubber stopper and two side arms, to flush through different concentrations of O_2 in N_2 before or after grinding. Before grinding, the light intensity was easily measured, and increased up to a maximum of 20 scale divisions on pure O_2. After grinding, the light intensity was less than half a scale division (unreadable), even after adding ATP. The maximum intensity occurred at a concentration of approximately 10% O_2. It was concluded that the reaction occurred more intensely in the organized cell than in its homogenate, and hence more oxygen was used up in the intact cells, resulting in a larger diffusion gradient for oxygen. The reason for the decrease in light production after disruption of the cell structure was not determined.

Studies on actin. I. The properties of G-ADP actin. TERU HAYASHI AND RAJA ROSENBLUTH.

The changes of state of the muscle protein actin can be described as a cycle of events to regenerate actin; *viz.*, G-ATP polymerizes to F-ADP with KCl and Mg, F-ADP depolymerizes to G-ADP in water, G-ADP converts to G-ATP by two methods: (a) Incubation in water with ATP, or (b) incubation in small amounts of Mg with CP and CPase. Method (a) is known to be a whole nuleotide replacement; method (b) is of interest because of the possibility of transphosphorylation of the bound ADP. As a start toward a study of this possibility, a study of the properties of G-ADP as a key substance in the cycle was undertaken.

G-ADP polymerizes upon the addition of KCl, $MgCl_2$ and ATP. The extent of the polymerizability is a measure of the activity of the protein. At 1° C., 20% of this activity is lost in an hour, and is found to be due to the loss of bound nucleotide. At 29° the loss is faster. The continuous presence of Dowex-1, which quantitatively removes *free* nucleotide, also hastens the loss of bound nucleotide. Mg ion inhibits this loss to varying degrees, depending on the concentration of the Mg.

G-ADP also polymerizes without ATP, simply upon the addition of KCl and Mg. At 29°, this polymerization is similar to that produced with ATP, both in rate and extent, but at 1° it is much slower. A tentative explanation for this difference is given. Of considerable interest is the fact that it is apparently unnecessary to dephosphorylate the nucleotide for polymerization *per se* unless, in this case, the ADP is being dephosphorylated to AMP.

A procedure for obtaining completely dissociated sponge cells. TOM HUMPHREYS, SUSIE HUMPHREYS AND A. A. MOSCONA.

When sponges are pressed through bolting cloth, most cells remain in clumps. Further straining excludes larger clumps, but small clusters remain. This, as well as the likelihood of cell-type selection, and the variability inherent in this procedure, restricts its usefulness in studies requiring dispersed cells. An effective method, based on the removal of calcium and magnesium, was therefore developed for complete dissociation of sponge tissue into viable cells. It has been extensively applied to aggregation studies on *Microciona prolifera*, but is effective also for *Cliona celata* and *Haliclona permollis*.

One gram of blotted sponge lobe tips was immersed in 80 ml. calcium- and magnesium-free sea water (CMF-SW) at 0° C. (NaCl, 27.0; KCl, 8.0; Na_2SO_4, 1.0; and $NaHCO_3$, 0.04 gm./l. H_2O, pH 7.2), cut into 2-mm. fragments, soaked for 30 minutes, and squeezed through #25 standard quality bolting cloth into 80 ml. fresh, cold CMF-SW. The resulting suspension contained single cells and small clumps at an approximate concentration of 15×10^6 cells/ml. The material was centrifuged at 1000 rpm and resuspended at a concentration of 20×10^6 cells/ml. in CMF-SW containing 0.1% trypsin (crystalline-lyophilized, Worthington), at 0° C. Fifteen-ml. aliquots of this suspension in 100-ml. beakers were rotated on a gyratory shaker at 80 rpm for 6 hours at 5° C. Three ml. of the resulting suspension were then transferred into a conical centrifuge tube, vigorously squirted 15 times through a pipette (¾-mm. orifice, 2-ml. bulb), spun down and resuspended in 1.5 ml. of sea water by pipetting.

The resulting suspension consisted of single cells. Maintained in stationary vessels, the cells formed aggregates which developed into sponges in a manner similar to that of conventionally dispersed sponge tissue.

It should be noted that trypsin did not noticeably assist in dispersion of the cells; however, for reasons yet unclear, trypsin-treated cells gave better aggregation results.

Rotation-mediated aggregation of dissociated sponge cells. TOM HUMPHREYS, SUSIE HUMPHREYS AND A. A. MOSCONA.

Aggregation of dissociated sponge cells provides a useful means for studying various cellular activities and interactions. Conventional "self-aggregation," in depending largely on cell migration and cell-sheet contraction, is susceptible to variables indirectly related to the pertinent problems, and is therefore inadequate for analysis of cell interactions. In the present procedure, cellular material is continuously maintained in suspension by rotation on a shaker and the cells brought in contact by the motion of the liquid. All experimental parameters are readily controllable and, under standard conditions, the results are consistent and amenable to quantitative assessment.

Aliquots of *Microciona prolifera* cell suspension (30×10^6 cells in 3 ml. sea water) dispensed into 25-ml. Erlenmeyer flasks are rotated for 24 hours on a shaker, ¾-inch diameter of rotation, 80 rpm at 25° C. The cells rapidly accumulate in the center of flasks and within minutes begin to cohere. After one hour all cells capable of cohesion are in small, irregular, loose clusters; these tend to fuse and reach final size at about 8 hours. By 24 hours the aggregates are round, compact, smooth, average 0.12 mm. in diameter, contain 1 to 2×10^5 cells, and are capable of development. Under these standard conditions, consistent, reproducible "aggregation patterns," defined by size distribution, shape and compactness of aggregates, are obtained. These are highly sensitive to experimental conditions, thus providing useful baselines for testing selected variables.

Using this procedure it has been found that: (1) aggregates are obtainable across a wide range of cell concentrations, (2) cells from different species have distinct aggregation patterns, (3) variations in dissociation procedure affect aggregation, (4) aggregation is temperature-sensitive in that cells remain dispersed at 5° C., although brought in contact by rotation, and form only small clusters at 10° C., (5) calcium is the only divalent cation sufficient for normal aggregation, (6) no cell cohesion occurs in the absence of divalent cations.

Delayed cleavage of fertilized Arbacia eggs after treatment with a "nitrogen mustard" or with formaldehyde. MILTON LEVY AND PEDDRICK WEIS.

Bis (beta chloroethyl) ethylamine (a nitrogen mustard gas) inhibits mitosis. The chemical action of the agent results in "alkylation" by replacement of active hydrogen by the alkylamine group with elimination of hydrogen chloride. The presence of two reactive groups allows the formation of cross-links between molecules, and seems to be essential for the biological effect of nitrogen mustards. Formaldehyde can also form cross-links.

Experiments (initiated in 1944) were done to define the actions of these agents on Arbacia eggs and sperm by following the progress of the first and sometimes the second cleavage. Egg sets and sperm were obtained by electrical stimulation, washed, and divided into batches. At appropriate times a reagent was added in 1/50 of the final volume. Treatments were terminated by sucking the supernatant off the settled eggs and washing to a nominal dilution of 2500-fold. Eggs were fixed and counted at intervals from each batch. To compensate for the variability among sets of eggs, the normal first cleavage time (55–68 minutes at 21°) of each set is taken as 100% and delays are given in terms of this time. For constant time of exposure the delay increases with concentration, but at 0.0009 *M* formaldehyde and 60-minute exposure the eggs cytolyze. The delay increases with time of exposure. Thus, using 0.00044 *M* formaldehyde for 10 minutes before fertilization produces no delay, but 15 minutes produces 13% delay and 60 minutes produces 75% delay.

Time of treatment in the cleavage cycle is very important. For the same treatment (5 minutes, 0.00072 *M* nitrogen mustard) the following data were obtained. Treatment before fertilization 75% delay, started just after fertilization 150% delay, 3 minutes later 115%, 12 minutes later 106%, 20 minutes, 40%, 24, 30, or 45 minutes no delay in the first cleavage but considerable delays in the second cleavage. The same treatment of sperm caused a 60% delay in the first cleavage of eggs fertilized with them.

Constancy of the pCO_2 in the ocean. W. F. LOOMIS AND W. F. LOOMIS, JR.

The carbon dioxide tension or pCO_2 of the ocean was studied under a variety of conditions. Using scuba, syringes were visually filled with bubble-free ocean samples taken at various

depths down to 60 feet. After collection, all samples were placed on ice and taken directly to the laboratory where their oxygen tension was determined by the Winkler method and their pCO_2 determined by a new and direct method that does not involve pH. Duplicate samples were taken at various depths and localities from April to August, sites including the ocean, fresh- and brackish-water lakes and a salt marsh. The diurnal cycle was studied by taking measurements at 3 PM, 9 PM, and 3 AM, in both fresh-water and ocean sites. The findings were compared to oxygen determinations made by the Oceanographic Institution at stations between Woods Hole and Bermuda at all depths down to the bottom of the ocean (5140 meters).

The highest pCO_2 found was 1.43% atm. This occurred in a 9° C. exolimnotic layer 15 feet down in a fresh-water lake in April. Since fully aerated water has a pCO_2 of 0.03% atm., this finding contrasts with the fact that no pCO_2 above 0.13% atm. was ever found in the ocean. Oceanographic data indicate a layer 1000 meters down that is about 55% saturated with air and hence has a calculated pCO_2 of around 0.3–0.4% atm. This would appear to be the maximum pCO_2 of the ocean as the per cent saturation with air rises both above and below this deep sounding layer. Since the diurnal cycle was found to be less than 0.1% atm. at 10 feet of depth in the ocean, it can be concluded that pCO_2 is an ecological variable that varies widely in fresh-water lakes but is held extremely constant in the ocean. This finding confirms the view that the oceans form a remarkably constant *milieu extericure*.

Carbonic anhydrase in the female reproductive tract of elasmobranch fishes. CECILIA LUTWAK-MANN.

Some years ago the author discovered the presence of carbonic anhydrase in the endometrial mucosa and placental tissue of various mammals, and in the uterine deciduoma of the rat. The occurrence of carbonic anhydrase was also established in certain parts of the oviduct of the egg-laying hen. These studies clearly demonstrated that carbonic anhydrase in the female reproductive tract depends upon hormones which control the function of the genital tract.

It was now decided to extend the study of the genital tract of certain elasmobranch fishes, at different stages of reproductive activity. The investigation was done on the vivparous-placental smooth dogfish (*Mustelus canis*), the viviparous-aplacental spiny dogfish (*Squalus acanthias*), and the oviparous skate (*Raja erinacea*). The genital tract of immature, or mature but non-pregnant, dogfishes showed none or negligible carbonic anhydrase activity. On the other hand, carbonic anhydrase was markedly active during early, and slightly less so in advanced gestation. The enzyme was localized in the lining and the wall of the uterus, the degree of activity being roughly equal to that found in the gills of these fishes. The oviducts and shell-glands were less active. In mature egg-laying skates the oviducts were the main site of carbonic anhydrase activity; the shell-glands were also active, and slight activity was demonstrable in the lining of the uterine folds. Practically no activity was detected in these organs in immature skates. Early dogfish embryos (4–7 cm.), yolk-sacs, or mature eggs of either dogfishes or skates were inactive. The ovarian tissue of these elasmobranchs was devoid of activity. In general, carbonic anhydrase values were highest in the tissues of the skate, and lowest in the spiny dogfish.

Reducing substances in blood of toadfish and catfish. PAUL FOLEY NACE.

It was apparent early in the investigation of alloxan diabetes in fish that pancreatic beta cell changes and blood Folin values followed a similar course. The use of the enzymatic Glucostat determination for glucose, in conjunction with the Folin-Malmros procedure for total reducing substances, confirmed this relationship for glucose but showed that another substance or substances participated even more markedly. Because the major concern of the investigation was the post-alloxan regeneration of the beta cells of the toadfish as compared to the non-regeneration in the catfish, it appeared necessary to examine these substances as possible factors in this species difference. In both species of fish, these substances accounted for most of the early change in Folin value. In toadfish, 60 minutes after alloxan injection, the Glucostat glucose had increased only 10 mg.% above control levels, while the total Folin reducing substances had increased nearly 100 mg.%. In man, Saifer found only 10% differences in non-diabetic patients and 20% differences in diabetics. The suggestion that alloxan or its

compounds were important components was not supported by the course of the blood changes or by the observation that egg-bearing females failed to show the post-alloxan increase in Folin value. The relationship of alloxan to uric acid led to assay of this compound in catfish blood after alloxan, with failure to demonstrate a significant contribution. Since Fashena had shown the importance of glutathione and glucuronic acid in the "saccharoid" fraction of human blood, both test tube and chromatographic studies of these substances were made, using catfish blood. No significant increases were found. In the toadfish, blood fructose appeared constant through post-alloxan hyperglycemia. N.I.H. A-1129 (C3).

Measurements of volume and composition of the swim bladder gas of toadfish (Opsanus tau). GEORGE POLGAR.

A pair of narrow lumen plastic tubes were sewn into both compartments of the swim bladder of unanesthetised toadfish through an abdominal incision. Their distal ends, sealed with caps, were left outside the abdomen after suturing the skin. Following this operation, and later at regular intervals, the caps were removed and, through a hypodermic needle, gas samples were withdrawn into syringes, the plungers of which were a Mylar-covered oxygen electrode and a Teflon-covered carbon dioxide electrode, respectively, for measuring the partial pressure of these gases. First the bladder was allowed to deflate to induce gas production. Subsequently the gas samples were reinjected into the bladder. The gas volume was measured before each analysis, with the fish in a water-filled pressure cooker serving as a plethysmograph. The volumes were calculated from Boyle's law. In seven fish, the initial oxygen concentration was between atmospheric (20.9%) and 49%, being higher in the anterior than in the posterior chamber in all but one fish. After deflation, a less concentrated oxygen was found in a small volume at first, but 18–48 hours after the operation, a peak was reached with a single maximum value of 86%. The changes of oxygen concentration were generally parallel in the two chambers. In one fish the carbon dioxide concentration rose in the anterior chamber prior to the rise in oxygen concentration. In another one, there was no such early peak. The concentration of carbon dioxide did not exceed 6% in any determination made on 7 fish, and the direction of its changes was usually the same as for oxygen.

These experiments indicate (1) that it is technically possible to sample both chambers of the swim bladder repeatedly under nearly physiological conditions; (2) some trends have been observed in the changes of composition and volume of the gases after deflation; and (3) a consistent difference was found between the concentrations in the two chambers.

An addition reaction of the α,β-unsaturated ether linkage of plasmalogens. MAURICE M. RAPPORT.

It was unexpectedly found that lipids containing high concentrations of plasmalogen (derived from Asterias eggs and Mytilus (whole animal)) lose their unsaturated ether linkage on standing for several days at 20–24° in chloroform-methanol solution. This instability was not seen when the original chloroform-methanol extract was exhaustively washed with water. Lipids which had lost the unsaturated ether linkage fully retained their capacity to generate higher fatty aldehyde as determined by p-nitrophenylhydrazone formation. These properties indicate an addition reaction of the unsaturated ether producing a mixed acetal (or hemi-acetal if the adduct is water). The reaction was observed in only one of two extracts of starfish eggs but in all four of the extracts of mussel that were studied. In unwashed chloroform-methanol extracts, the unsaturated ether was relatively stable. It has been suggested that the function of plasmalogen may be related to its capacity to add water-soluble compounds at the activated double bond. While the observations made are consistent with this hypothesis, interpretation must await a more complete knowledge of the reactants.

Inhibition of fertilization of Asterias, Spisula and Chaetopterus eggs by Arbacia dermal secretion. HERBERT SCHUEL AND CHARLES B. METZ.

Upon brief (60-second) exposure to tap water the adult *Arbacia* releases a yellowish-green fluid called dermal secretion (DS), which inhibits fertilization of *Arbacia* eggs. The

DS appears to inactivate some egg substance that is essential for fertilization. In order to determine if this were a highly specific effect, the *Arbacia* DS was tested for action on fertilization in other species.

Eggs of the starfish *Asterias forbesi* did not fertilize in the presence of DS. Eggs washed from DS exhibited an almost complete loss of fecundity; *i.e.*, even when very high sperm concentrations (0.2%) fertilized few of the eggs. Exposure of eggs to DS subsequent to elevation of the fertilization membrane did not inhibit cleavage. Sperm washed from DS fertilized eggs as readily as sperm washed from sea water. These results are similar to those that have been obtained with *Arbacia* eggs and sperm.

In the presence of DS, fertilization of the eggs of the clam *Spisula solidissima* was inhibited because the sperm were killed. Eggs washed out of DS fertilized as well as the controls (the germinal vesicle broke down), but the DS-treated eggs did not divide. Exposure of eggs to DS after fertilization resulted in an inhibition of cleavage that could be partly reversed upon return to sea water. High concentrations of *Spisula* sperm (10%) absorbed and removed from solution the fertilization and cleavage inhibitors.

Preliminary experiments indicate that DS inhibited the fertilization of *Chaetopterus pergamentaceus* eggs. This may be the result of action on both the eggs and the sperm. Eggs exposed to DS after fertilization did not divide.

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Further studies on the antimitotic action of heavy water. WILLIAM SPINDEL AND P. R. GROSS.

The immersion of fertilized sea urchin eggs in sea waters reconstituted with excess deuterium results in delays in, or blockade of, mitosis and cytokinesis. D_2O concentrations up to 30–40% cause relatively simple delays in the cleavage cycle, although the incremental periods may be very long. Higher concentrations change the kinetics of cleavage as well, and permit smaller total numbers of eggs to enter mitosis. At concentrations higher than 65–70%, cell division is totally blocked. These effects are entirely reversible upon removal of the deuterium-enriched medium and replacement with sea water, except in cases where fertilized eggs have remained blocked in D_2O for several hours. The blockade is related to the mitotic cycle in general and not to nuclear events unique to the first cleavage cycle, for eggs can be blocked in the same way during cleavage cycles subsequent to the first. Reproduction and division of the cell centers, and perhaps of the genetic material, appear to continue during the blockade of karyo- and cytokinesis, since cells which have remained arrested in D_2O for long periods divide directly into multiple-celled forms when washed with normal water. The inhibition of fertilization itself is an effect upon the egg, not the sperm, since motility and fertilizing power are retained for significantly long periods after immersion of spermatozoa in D_2O. Current notions concerning the mechanism of antimitotic action by D_2O invoke changes in the normal hydrogen-bond cross-linking of cytoplasmic structure-forming macromolecules and particulates. In this connection, it has been found that the cytoplasmic "viscosity," measured by particle sedimentation, rises exponentially with increasing deuteration of the medium, reaching, in 95% D_2O, values more than sixteen times as great as those characteristic of sea water controls. This type of effect is also observed in fertilized eggs. The simple cleavage delay in 30% D_2O falls sharply in absolute length as the temperature is raised from 15 to 29° C. The effect of this is to make the delay a constant fraction of the cleavage interval in the temperature range indicated.

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Comparative studies of malic and glutamic dehydrogenases. CLAUDE A. VILLEE.

The studies of Kaplan *et al.* (*Science* 131: 392, 1960) have shown that lactic dehydrogenases from different tissues of the same animal may react at different rates with the several pyridine nucleotide analogs. In addition, lactic dehydrogenases from different animals have reaction rates with the several analogs in patterns which indicate evolutionary relationships: closely

related animals have lactic dehydrogenases with similar patterns of reaction rates with these analogs, whereas unrelated animals have lactic dehydrogenases with markedly different patterns. To determine whether other dehydrogenases show comparable differences, and give patterns of reactivity that correspond to those for lactic dehydrogenases, tissues from a variety of invertebrate and vertebrate animals were homogenized and centrifuged in the cold, to sediment particulate matter. Aliquots of the supernatant fluid were tested for malic and glutamic dehydrogenase activity with diphosphopyridine nucleotide (DPN), 3-acetyl pyridine DPN (APDPN), 3-pyridine aldehyde DPN (PADPN), deamino DPN, and thionicotinamide DPN (TNDPN). Reaction rates were measured spectrophotometrically at the wave-length of maximum absorption of the reduced pyridine nucleotide or analog. Control cuvettes contained the complete system minus the substrate (malate or glutamate), to correct for reduction of pyridine nucleotides due to other enzymes and endogenous substrates. The patterns of relative reaction rates with the different analogs for malic and glutamic dehydrogenases are quite different. The rates with DPN and deamino DPN are generally similar but the enzymes tested reacted much more rapidly with the other three analogs than with DPN. The differences which characterize each tissue are based primarily on the rates with APDPN, PADPN, and TNDPN. Malic dehydrogenases of comparable tissues, *e.g.*, ovary, of related animals, *e.g.*, *Arbacia, Strongylocentrotus, Asterias, Echinarachnius* and *Thyone*, were more similar than those of different tissues, *e.g.*, ovary, testis, gut, and muscle, from the same species. Although the absolute patterns of reactivity are different for malic, glutamic and lactic dehydrogenase, evolutionary relationships are discernible on the basis of any of these.

Experiments with Ca^{45} in marine egg cells. FLOYD J. WIERCINSKI AND JAMES K. TAYLOR.

The egg cells of *Arbacia punctulata* and *Spisula solidissima* were used in various experiments with Ca^{45}. Egg cells were exposed to sodium citrate and EDTA, then Ca^{45} was applied. With ultraviolet light and x-ray Ca^{45} was present in the medium during the exposure. Also, cells were incubated in calcium-free sea water with known amounts of Ca^{45}. Samples of dried eggs and dried medium on planchets counted with a beta detector gave data that were in the range of the standard deviation.

Observations were made with the cells crowded in Ca^{45} solutions of 0.8 mm. thickness by means of a beta gas flow detector coupled to a rate meter and a continuous recording device. Under normal conditions there was very little, if any, uptake of Ca^{45}. Cell calcium is in equilibrium with that found in sea water. Long exposure to ultraviolet light, 150 Kr of x-ray, sodium citrate and EDTA showed varied uptake of Ca^{45} by a decrease in the counting rate.

Autoradiographic stripping film technique on whole mounts, sectioned eggs and ovaries of Arbacia showed the localization of Ca^{45}. Arbacia were grown in Ca^{45} sea water for ten days. Sections of the ovary showed that the immature eggs were rich in Ca^{45}. These data indicate that calcium is incorporated into the cell structure before the maturity of the egg cell. Silver grain counts were made on whole mounts of Arbacia eggs from the unfertilized stage to the blastula stage. A progressive increase in the number of grain counts per unit area on the jelly coat and the cortex had been observed during development. Staining techniques indicate that the cortex is probably a layer of calcium-nucleic acid complexes. Many slides have been prepared for further analysis.

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A function for the nucleolus. W. S. VINCENT AND E. BALTUS.

The nucleolus has long been associated with concepts of protein synthesis. Caspersson and Brachet recognized that the basophilia of nucleoli was due to RNA, and postulated that the RNA portion of the nucleolus might direct protein synthesis. Recent biochemical studies have shown that at least two types of RNA are required in the synthesis of protein. One of these is bound to the RNP granules of the cytoplasm (granule RNA); the other of the RNA's is "soluble," and has been shown to act as an activation agent for amino acids through the addition of terminal nucleotides.

Our analyses have shown that the starfish oocyte nucleolus contains an RNA fraction which adds (terminally) two cytidylic acid residues. This fraction, when exposed *in situ* to C^{14}-leucine, binds the leucine with an alkali labile bond. Attempts to extract this RNA by the cold *M* NaCl technique of Goldthwait resulted in the solubilization of 80–85% of the nucleolus. Part of the RNA contained in this fraction is tightly bound to the protein, the rest remains in solution after the protein is precipitated by dilution to 0.1 *M* NaCl. The leucine appears to be initially bound to the free RNA; upon further incubation the specific activity of the protein-bound RNA increases, while that of the soluble RNA decreases.

Analytical ultracentrifugation of the cold salt extract reveals the presence of a large homogeneous peak with a sedimentation coefficient of 4.5 S. The C^{14}-leucine is associated with the RNA attached to this protein.

The data reported here, and many other observations in the literature, are consistent with the following hypothesis: A major function of the nucleolus is the provision of "soluble" or "activation" RNA for use in the synthesis of cellular proteins. The "activation" RNA provided by the nucleolus is bound to a carrier protein, likewise nucleolar in origin; this protein is considered to be essential for the integrity of the RNA.

Senior Research Fellow, USPHS, and Chargée de Recherches du Fonds National de la Recherche Scientifique, Belge, respectively.

The "black" eye colors in Mormoniella. P. W. Whiting.

Many mutations have occurred changing dark brown wild-type eyes to brighter colors—red, orange, yellow, peach, white. *Very few* mutants have been found with eyes darker than wild-type. These "blacks" undoubtedly occur much more frequently than the records show, possibly as frequently as the brighter colors, but are difficult to detect. They have been divided into two groups—the *bk* blacks and the *eb* (ebony) blacks. The latter have no effect on scarlet (*st*) or on vermilion (*vm*), *st eb* and *vm eb* being scarlet and vermilion, respectively. Dahlia, a dark red, appears black in combination with ebony (*da eb*). The action of the *bk* blacks appears to be to prevent the development of the bright pigments, leaving the black pigments unaffected. Thus scarlet, having no (or very little) black pigment, is changed to white (*st bk*); vermilion, with intermediate amount of black pigment, becomes light lavender (*vm bk*) and dahlia, with much black pigment, becomes dark lavender (*da bk*). It is considered that the lavender chroma is due to a structural condition overlying black pigment, as in the feathers of Blue Andalusian fowl. Both the *bk* blacks and the *eb* blacks change orange and peach to white (*or bk, or eb, pe bk, pe eb*). It is expected that certain genetics tests now under way clarify the seemingly contradictory effects in the ebony combinations with the bright color genes. Irradiation of dahlia, orange or peach stocks should show more "black" mutants than irradiation of wild type, scarlet or vermilion. Homologies and linkage relationships are being determined. There are at least three loci, with multiple alleles at one of them. (Work supported under contract AT(30-1)-1471 with the U. S. Atomic Energy Commission.)

PAPERS READ BY TITLE

The ionic composition of squid photoreceptors. R. G. Adams and W. A. Hagins.

Though the electrical activity of many retinas has been studied, little seems known of the electrochemical basis of the currents and voltages so frequently seen. The squid retina is a favorable tissue for use in estimating the chemical composition of photoreceptors, since at least 90% of the retinal cellular bulk consists of the receptors and their cell processes. Opened squid eyes were dark-adapted and equilibrated at 10° C. in oxygenated, sulfate-free artificial sea water for 2–3 hours in the dark. The retinas were then freed from their scleras in dim red light, blotted and analyzed. They contained 79% water, 35% of which was accessible to inulin and is thus considered to be "extracellular." Allowing for the extracellular fluid, the mean ionic composition of the retinal cells was: K^+, 300 mM; Na^+, 110 mM; Cl^-, 140 mM. Since the artificial sea water contained 10 mM K^+, 430 mM Na^+, and 571 mM Cl^-, the Nernst potentials for the ions were: Na^+, 34 mV; Cl^-, −35 mV; K^+, −93 mV. The ionic composition of the cells of the squid retina is thus grossly similar to that of many other types of cells. However,

the failure of agreement between the K^+ and Cl^- potentials implies that the cell membranes are not equally permeable to both ions, or else the receptors are not fully equilibrated after 3 hours in ASW. Nevertheless it is clear that electrochemical batteries are available in the photoreceptor membranes for production of electrolytic currents of the type seen in other cells, such as nerve and muscle.

Transport of nutrients in the holothurian Leptosynapta inhaerens. A. S. D'Agostino and A. Farmanfarmaian.

Several investigators have reported that the digestive tube of holothurians, although permeable to water in both directions, is completely impermeable to a variety of substances tested. These experiments, in addition to histological observations, have given rise to the hypothesis that the transport of nutrients in these organisms is largely or entirely mediated by amebocytes which occur abundantly in various tissues. The mode of nutrient transport in the echinoderms in general is, furthermore, in confusion.

To elucidate some aspects of this problem a number of experiments were carried out, using the holothurian *Leptosynapta inhaerens.*

The body wall was cut from a point just posterior to the mouth to a point just anterior to the anus. The anus was ligatured and the mouth cannulated. The preparation was then rinsed in filtered sea water and suspended in 5 ml. of the desirable isotonic solution. Under these conditions both the body wall and the digestive tube continue to exhibit peristalsis for 8 hours in sea water. Punctures in the gut wall could readily be detected and the preparation discarded when a suspension of neutral red in sea water was introduced into the digestive tube. Experiments involving the use of various indicators showed the pH of the esophagus, stomach and the intestine to be 6.8, 6.8, and 8.0, respectively. In a number of experiments 2000–3000 μg. of dextrose in sea water solutions were introduced into the digestive tract, and at time intervals the outside fluid tested for dextrose. The results of these experiments show that there is a passage of dextrose from the gut to the outside fluid at a rate of 16–17 μg./min. at 20°–23° C. Under these conditions 70–80% of the sugar appears in the outside fluid within two hours. When parallel experiments were carried out in isotonic $MgCl_2$ solution, in which the animal was relaxed, the rate dropped to 8 μg./min. These results indicate that contrary to earlier reports for other holothurians, the digestive tube of *Leptosynapta inhaerens* does allow direct passage of dextrose to the outside, and that mechanisms other than simple diffusion may be involved.

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Directional movements of the intertidal snail, Littorina littorea. Douglas G. Alexander.

Individuals of *Littorina littorea* are located within a specific intertidal region. When they are displaced above or below this region they move back into the preferred zone. Through a marking and recapture program the intertidal movements were recorded. Individual snails were marked with quick drying paint during low tide. After varying lengths of time subsequent movements were determined by triangulation.

With the exception of the forms displaced above the spring high tide level, all displaced forms moved in a direction which compensated for their displacement and brought them back into the normal zone of distribution. Individuals released within the zone of normal distribution displayed random movement at a reduced rate compared to the directional movements of displaced forms. This rate of movement within the zone of normal distribution became further reduced after a series of tidal cycles. The initial movement may have been due to the abnormal concentration of marked snails at the release point, and their subsequent reduction in movement may have been the result of their integration into the naturally existing population. Snails moved only when covered with water. Animals displaced above the tide level failed to move, indicating that the snails are inactive when exposed to the drying conditions of low tide. The forms displaced below normal distribution were placed upon a different type of sediment, consisting of fine silt. The trails indicated a rather direct movement toward the region of normal occurrence.

This work was part of the student training program of the Marine Ecology Course and supported in part by the Department of Zoology, University of North Carolina.

Observations on the ribosomes of sea urchins. WILLIAM S. ALLISON AND ELOISE E. CLARK.

Preliminary studies, conducted primarily by ultracentrifugal methods, were undertaken in order to determine conditions for isolating and stabilizing the ribosomes of the sea urchin. Unfertilized eggs and embryos in various stages of development (mid-gastrulae, 48-, 60- and 65-hour plutei) were chosen as the material to be used. Examination of both the crude extracts of cells and partially purified preparations of the fast sedimenting components revealed that the conditions required for the stabilization of the larger ribosomes of the sea urchin were not the same as those reported for other organisms. It has been found that the addition of magnesium to the extracting solvent is necessary for maintaining the structural integrity of the so-called 70S particles of *E. coli* or the 80S particles found in yeast and various mammalian tissues. This does not appear to be the case for extracts prepared from sea urchin eggs or embryos. When extracts of either unfertilized eggs or plutei were prepared in the absence of added magnesium (using 0.01 *M* phosphate buffer at pH 7.0, or with this buffer containing 0.002 *M* ethylene diamine tetraacetate), the schlieren pattern in the analytical ultracentrifuge showed a major component which had a sedimentation coefficient of 70–75S. When comparable extracts were prepared using either Tris or phosphate buffer and with varying concentrations of magnesium (from 0.001 to 0.1 *M*), the fastest component observed sedimented at 23–26S, indicating that the larger components had been degraded. Partial purification of the 75S component by means of cycles of alternate low- and high-speed centrifugation, followed by suspending the particles in a buffer containing 0.1 *M* KCl, led to the disappearance of the 75S component and the appearance of components which were observed to sediment at 43 and 24S. It is of interest that whereas the classes of particles from this organism were similar to those observed in other forms, the properties of stability were found to be somewhat different.

Regeneration of the cardiac stomach in Asterias forbesi. JOHN MAXWELL ANDERSON.

Starfishes that feed by everting the cardiac stomach continually place this delicate and essential feeding organ in hazardous situations; it would seem that ability to repair or regenerate the stomach in case of loss or damage would be of great survival value to the starfish. In *Asterias*, eversion can be induced by soaking the animal in $MgCl_2$ solution and then squeezing one or more of the rays. The everted stomach can be excised by cutting it across at the peristome and just below the pyloric stomach and severing its connections with the retractor harness. Study of a series of animals thus prepared and sacrificed at weekly intervals has provided the following information: (1) within three weeks, the integrity of the gut is reestablished and the wall of the regenerated organ contains all the normal tissue layers; (2) the new stomach forms by upward growth of a sleeve of tissue from the peristome, making contact with the retractor nodule in each ray and eventually joining the pyloric stomach; (3) establishment of contact with the retractor nodule apparently induces formation of a pouch-like enlargement in the adjacent sector of the stomach—where contact is missed, as sometimes happens, no pouch is formed; (4) at three weeks, the wall of each pouch has begun to form a small but normal pattern of gutters and folds, with normal histological differentiation in the epithelium—multiflagellated cells with huge, dense, spindle-shaped nuclei over the folds, single-flagellated cells with granular, ovoid nuclei in the gutters; (5) between three and five weeks, the intrinsic retractor fibers invade the connective-tissue layer, growing downward beneath the branching gutters to form the normal corresponding pattern; (6) although the stomach is small and its normal specializations are present in only rudimentary condition, the starfish is capable of ingesting and digesting prey (amphipods, small snails) within the fourth to fifth postoperative week. The regenerating cardiac stomach provides valuable material for study of early stages in histological differentiation.

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A day-to-day relationship between oxidative metabolism and world-wide geomagnetic activity. FRANKLIN H. BARNWELL.

O_2-consumption of mud snails, *Nassarius obsoletus*, was measured continuously with Brown recording respirometers from June 19 to July 19, 1959, in Woods Hole, Mass. There was found to be a remarkable similarity between the day-to-day changes in mean daily metabolic rate and the form of the day-to-day changes in magnetic activity as measured by the international magnetic character figure, C. Throughout the period of study the metabolic activity on any given day, n, was clearly related to the intensity of magnetic disturbance on the first and second days thereafter, $n+1$ and $n+2$. This is indicated by the coefficients of correlation, -0.550 for the relationship to $n+1$ and -0.539, to $n+2$. Examination of other lag or lead relationships revealed little or no agreement between the two series. Twenty-nine coefficients of correlation for other temporal relationships of metabolic activity on day n to magnetic activity, from day $n-15$ to day $n+15$, had a mean value of -0.068 and standard error of ± 0.028. The coefficient of correlation for each day $n+1$ and day $n+2$ was significantly different from this population, $p < .005$. Such a correlation suggests that in rigorously controlled, constant conditions metabolic rate of the snails was reflecting a response to a pervasive factor related to solar, and subsequent world-wide geomagnetic, activity.

This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-122803.

Comparative electrophysiology of supramedullary neurons. M. V. L. BENNETT.

The supramedullary neurons (SMC) of *Lophius piscatorius* (angler fish) and *Tautogolabrus adiposus* (cunner) have been studied with microelectrode recordings. In both forms, a number of similarities were found to the SMC of *Spheroides maculatus* (puffer; *cf.* Bennett *et al., J. Gen. Physiol.*, 1959, 43: 159). Spikes could be evoked by brief stimuli to the spinal cord or cranial nerves, or by tactile stimulation of the skin. These indirectly evoked impulses arise in the axons, some distance from the soma, but subsequently invade the latter. An inflection on the rising phase of the spike recorded in the soma indicates delay at the axon hillock. The impulses are initiated by two kinds of depolarization arising in the axon. One is long-lasting, graded, and presumably of synaptic origin. The other is due to reciprocal excitatory connections between the cells. These connections are electrotonic, since hyperpolarization as well as depolarization is transmitted during intracellularly applied testing currents. As a result, when one cell fires, it depolarizes adjacent cells and all cells tend to fire synchronously. Thus, on indirect stimulation, with a near-threshold stimulus, all cells tend to fire, or not to fire, and in multiple discharges to a single stimulus all cells tend to produce the same number of spikes. However, the synchronization is less effective than in *Spheroides*, and frequently the number of spikes differed in different cells. The electrophysiological similarities found in the three forms indicate that the supramedullary neurons are homologous in all. However, the function of the SMC remains unknown. In *Lophius* the impulses of the SMC were shown to pass out the dorsal roots, but not the ventral. Thus, whatever their function, it is probably similar in *Lophius* and *Spheroides*.

Electron microscopic studies of the reflecting structures of elasmobranch and teleost eyes. M. H. BERNSTEIN AND T. S. DIETRICH.

Comparative observations have been made of the elasmobranch tapetum and teleost argentea to correlate their fine structure and reflecting functions. Electron microscopic preparations of three elasmobranchs: smooth dogfish (*Mustelus canis*), sting ray (*Dasyatis centrura*), and basking shark (*Cetorhinus maxime*), and a teleost (*Tautoga onitis*) were used. Histological observations were correlated with the electron microscopy.

All of the elasmobranchs examined were similar. The tapetum is formed by modified choroidal cells and covers the whole outer surface of the retina. Cellular processes containing melanin granules interdigitate in regular alternation with elongate crystals (presumably guanine) and their associated cytoplasmic extensions. In the light-adapted eye, the inner aspect of the crystalline plates is covered by one or more layers of melanin granules. In

specimens dark-adapted for 12 hours, the melanin granules have migrated away from the retina so as to expose more of the crystalline material to the incoming light.

The angle between the crystalline plates and the optical axis approximates 45 degrees over much of the eye. In the area of the optic pit the plates are perpendicular to the axis. Toward the anterior portions of the eye, the crystals remain parallel to one another, but also become parallel to the incoming light. That is, a change is observed between the angle of the plates and the light axis. The orientation of the plates with respect to the light axis changes from 90 degrees at the optic pit to 0 degrees near the ciliary body. It has also been observed that the relative amount of crystalline material appears to be greater in the posterior pole of the eye.

The argentea of *Tautoga onitis* lies between the choroid and cartilagenous sclera. Bundles of parallel crystals lie in a connective tissue net. They lack the orderly relationship seen in the tapetum. This might be expected since the camouflaging function of the argentea does not require optically true reflection.

In addition to a confirmation of the above observations, work is now being done on the migration of elongated pigment granules in the retinal epithelium of *T. onitis* during dark adaptation.

The morphology of starfish spermatozoa. M. H. Bernstein and L. G. Fehrenbaker.

Electron microscopic examination of thin sections of the starfish (*Asterias forbesi*) spermatozoa shows a roughly spherical, slightly flattened head composed of an acrosome, nucleus and mid-piece. The acrosome is a complex mushroom-shaped structure contained within a bowl-shaped depression, as demonstrated by J. C. Dan, with the inferior portion elongating into a sac. The acrosomal depression is defined by a strong osmiophilic membrane. The acrosome body is faintly osmiophilic and contains a central electron-dense star. The star has a spherical central region with seven projections lying in a common plane. A crescentic electron-dense mass surrounds the acrosome body in its equatorial region. Posteriorly, the limiting membrane of the acrosome is thickened into a plate which separates the narrow caudal extension from the main mass of the acrosome body. A dense osmiophilic knob is attached to the plate and hangs down into the posterior sac.

The genetic or chromosomal material of the sperm head forms a cup around the posterior aspect of the acrosome. The nuclear material is granular and electron-dense in character.

The crescent-shaped mid-piece in turn holds the nuclear material of the spermatozoan on its superior concave surface. The mid-piece appears to be a single ring mitrochondrion with a central hole for the insertion of the tail. The mid-piece is separated from the head by a thin line of moderately electron-dense amorphous material, which thickens to a large mass at the periphery of the head mid-piece connection.

The sperm tail is composed of a central core of two filaments surrounded by nine pairs of filaments. The central core is lost at the entry of the tail into the opening of the mid-piece, as the remaining filaments extend into the mid-piece in the region of the tail attachment to form the proximal or ring centriole of the mid-piece.

A common membrane encloses the whole spermatozoan. It fuses anteriorly with the acrosome membrane, which in turn splits to form two membranes on either side of the outer dense crescent of the acrosome. The sperm membrane continues over the nucleus and mid-piece and is reflected onto the tail.

Hexose and pentose utilization by mackerel erythrocytes. T. A. Borgese and James W. Green.

The object of the present study was to examine the anaerobic metabolism of the mackerel red blood cell. A glycolytic pathway exists in these nucleated erythrocytes, as measured by the disappearance of glucose and the production of lactic acid at 15° C. and pH 7.6. It was demonstrated that the glycolytic quotient was dependent upon the level of phosphate in the suspension medium. When the phosphate concentration ranged from 13 to 80 μM/ml. suspension, with an hematocrit of 13%, the glycolytic quotient dropped from 0.47 to 0.30, respectively, suggesting that an increasingly greater dependence was being placed on respiration.

On the addition of IAA or KCN there resulted a decrease in the rate of glucose disappearance. Lactate production was virtually abolished with 10 μM KCN/ml. suspension (hematocrit 15%). IAA resulted in a reduction in lactic acid formation of about 64% relative to control suspensions.

The production of lactate from ribose appears to rival, and in at least one experiment exceeded, the production of lactate from glucose. The use of KCN in ribose supplemented suspensions also decreased the rate of lactate formation to approximately 70% of the control. With the control cells the rate of ribose utilization was best represented by a biphasic curve. The more rapid initial phase had a rate of 0.61 μM ribose used/ml. suspension/hr. The second phase was considerably slower, 0.12 μM/ml./hr. The hematocrit in this experiment was 15%. Ribose disappearance in the presence of KCN was not measured since additional experiments showed that cyanide interfered with the orcinol reaction for pentoses. The apparent stimulation of ribose used was not a real effect but simply a linear function of the KCN concentration.

The overall results suggest that a glycolytic pathway exists in mackerel erythrocytes, and that pentose is metabolized to lactate via a path comparable to the hexose monophosphate shunt, which is the main alternative to the glycolytic pathway of carbohydrate metabolism in animal tissues.

Nature and action of the fertilization inhibitor from Fucus. J. M. BRANHAM AND C. B. METZ.

Certain extracts of the brown alga, *Fucus vesiculosus,* inhibit fertilization and the fertilizin agglutination of echinoderm sperm. The chemical nature and mode of action of the inhibitor(s) have been further investigated.

Activity was found in the ethanol-soluble fraction of water extracts of the alga. The inhibiting material was precipitated with lead acetate and recovered from the precipitate. Esping (1957) concluded that such preparations contained polyphenolic substances. Color tests (F^{+++}, $K_2Fe(Cn)_6$, I_2, OH^-), precipitation reactions (heavy metals, $Cr_2O_7^=$, $(NH_4)_2MoO_4$, CaO, streptomycin, gelatin, agar, and in salt solutions), solubilities (soluble in methanol, ethanol, butanol, insoluble in xylene, acetone, ether), and nondializability suggest that the activity may be associated with tannins. Tannic acid (Merck) showed similar biological activity when tested on *Arbacia punctulata* gametes.

Fertilization inhibition resulted from an action on eggs, for sperm washed from inhibiting concentrations of the extracts were capable of fertilizing eggs, whereas membrane formation and cleavage (*i.e.,* fertilization) were irreversibly inhibited by exposure of eggs to *Fucus* extracts. If fertilized eggs were exposed during membrane elevation, incomplete membranes resulted and cleavage was greatly altered. Eggs with complete membranes cleaved normally in the extracts. Trypsin digestion (one hour in 0.05%) did not restore fertilizability (cleavage) to extract treated eggs, although trypsin treatment prior to exposure to *Fucus* extracts rendered eggs insensitive to the inhibiting action of the *Fucus* extracts.

Fucus extracts inhibited both fertilization and fertilizin agglutination of sperm (where applicable) in all species tested. Thus, the action of the agents is not specific.

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Endocrine control of the chromatophores of the zoeae of the prawn, Palaemonetes vulgaris. EDMUND S. BROCH.

Work done on the color adaptation of the zoeae of decapod crustaceans has indicated that the color responses differ substantially from those of the adults: specifically that the chromatophores respond directly to light and show no background response. An exception to this was a slight albedo response noted by Keeble and Gamble in second zoea of *Palaemonetes.* This was attributed to the extension of the eyestalk at that stage. It has been reported that chromatophores of first zoea do not respond to adult chromatophorotropins. There is no mention in the literature concerning retinal pigment movements in zoeae, as has been reported in adult decapods. Larvae of *Palaemonetes vulgaris* were reared in the laboratory through the eight zoea stages. Animals from one brood were used in each experiment, to insure similar stages of development and minimal genetic variation. The study was made only of the

monochromatic red chromatophores which make up the secondary system, as defined by Keeble and Gamble. In all experiments conducted, the chromatophores were in a concentrated state when animals were on a white background and completely expanded on a black background, thus displaying a true albedo response. This response occurred from the time of hatching to the eighth zoea stage. First stage zoeae with sessile eyes responded as completely and rapidly to different backgrounds as did second stage zoeae with eyestalks. The red chromatophores on an excised thoracic section of a second stage zoea, kept in crustacean tissue culture medium, contracted upon addition of brain extract, expanded upon addition of abdominal nerve cord extract, and contracted when eyestalk extract was added. The distal retinal pigment of animals kept in darkness did not move to a dark-adapted state. These observations indicate that endocrine control of the chromatophores in the zoea of *Palaemonetes vulgaris* is comparable to that of the adult.

Magnetic field strength and organismic orientation. FRANK A. BROWN, JR. AND FRANKLIN H. BARNWELL.

Snails, *Nassarius obsoletus*, were observed as they emerged from a magnetic-south-directed corridor into a symmetrically illuminated field under alternating conditions of the earth's magnetic field alone and experimentally modified magnetic fields. The characteristics of orientation of the snails were observed during their first 3 cm. of free movement. The experimental magnetic fields consisted of a reversed direction of the horizontal component of the field, and with a series of 8 horizontal strengths: 0.04, 0.1, 0.2, 0.4, 0.8, 2, 5, and 10 gausses. For each experiment 5 snails emerged alternately in the earth's horizontal field of 0.2 gauss and in an experimental field, first in an ascending and then in a descending order of strength. Four such experiments were always run contemporaneously, and always between 9 AM and noon to obviate influence of the known daily rhythm in orientation. Fifty groups of four experiments were conducted, uniformly distributed over a period of exactly two synodic months. The experimentally reversed magnetic fields resulted in an altered mean path of the snails. When the strength of the horizontal component (H) of the reversed field differed by no more than a factor of 3 to 4 (0.2, 0.4, 0.8 and 2 gausses) from the earth's total field, F (*ca.* 0.6 gauss), there was a synodic monthly cycle of response with right-turning over *full moon* and left-turning the remainder of the month. For reversed fields with H differing by a factor of 4 to 15 from the earth's F (0.04, 0.1, 5 and 10 gausses), there was similarly a synodic monthly cycle of response but now 180° out of phase, with right-turning over *new moon* and left-turning the rest of the month.

This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-1228-03.

A relationship between photic and magnetic response in snails. FRANK A. BROWN, JR. AND ANNICK HUTTRER.

Mud-snails, *Nassarius obsoletus*, were permitted to emerge from a magnetic-south-directed corridor over a polar grid such that the mean angle of right or left turning could be ascertained during their first 3 cm. of free movement. The conditions under which the snails emerged were the following, in order: (1) in the earth's magnetic field into a symmetrically illuminated white field, (2) in a field black to left and white to right, (3) same as the preceding but in a 5-gauss horizontal magnetic field oriented in reverse to the earth's, (4) same as (1), (5) in a field black to right and white to left, (6) same as 5 but in a 5-gauss horizontal magnetic field oriented as in (3). For each experimental run the series was repeated, with 5 snails in each condition, four times. Such experiments were carried out 18 times, 6 during mornings 8–10 AM, 6 during afternoons 1:30–3:30 PM, and 6 during evenings 6–9 PM. Incident illumination in all cases was 150 lux from above, 20 lux from the black side and 60 lux from the white. The snails showed a distinct positive phototaxis; when the black field was to the left the mean path was $+15.9 \pm 1.96°$, and when to the right, $-18.4 \pm 2.52°$. The deviation in path in response to the asymmetrical light field varied from experiment to experiment. There was, however, a distinct correlation between the strength of the response to the asymmetrical light field and the effect of the 5-gauss magnetic field. For weaker light responses ($< 15°$),

either + or −, the magnet produced left-turning and for stronger light responses (15° to 42°), right-turning. The coefficient of correlation between strength of light response and strength of magnet response was +0.40 ($t = 3.6$, $N = 72$, $P < 0.001$).

This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-1228-03.

A "compass-direction effect" for snails in constant conditions, and its lunar modulation. FRANK A. BROWN, JR. AND H. MARGUERITE WEBB.

At the same time each day, to avoid the daily rhythm of mean path of orientation, *Nassarius* were allowed to emerge from narrow corridors directed in four compass directions, North, East, South and West, into a symmetrically illuminated field. In four contemporaneously conducted experiments a total of 40 snails emerged in each direction between the hours 1 and 4 PM. For each of two synodic months there was minimum left turning, or maximum right turning when the snails were emerging northward. An analysis of the data indicated, however, that there was a synodic monthly modulation in the form of the change in mean path with compass direction. For both months, from first to third lunar quarter the relationship was bimodal with two peaks of greater right turning, one for the north- and the other for the south-directed snails, and for the semimonth from third to first quarter there was a unimodal pattern of fluctuation with maximum right turning in northerly directed snails and maximum left turning in southerly directed ones. The ranges of differences for the two semimonths following new moon (3.4° and 3.8°) were only about half those observed for the two semimonths following full moon (7.2° and 7.4°). That magnetic field clearly plays some role in this organismic "direction-effect" was evident in comparing the relative effects of reversing the field of the north- and south-bound snails with, consecutively, 0.4 and 2 gauss horizontal fields. For the north-bound snails the response to the weaker field lay 2.54° to the *left* of that for the stronger field, and, to the contrary, for the south-bound snails the response to the weaker field lay 2.92° to the *right* of that of the stronger field.

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Some effects of lysergic acid diethylamide and related agents on embryonic heart rate in Fundulus. JOSEPH A. BURKE.

In an attempt to ascertain the earliest measurable effect of lysergic acid diethylamide (LSD-25) and related psychotomimetic drugs on some specific organ, 25 *Fundulus* eggs were grown in 50-ml. solutions of lysergic acid diethylamide, 1-methyl-lysergic acid butanolamide (UML-491), yohimbine and serotonin, respectively, in sea water held in finger bowls at 21° C. In concentrations of 100, 50, 10 and 1 μg./ml., LSD-25 and yohimbine depressed heart rate. In LSD-25, development of a functional heart was delayed at least 48 hours. When a pulse was obtained in test embryos it varied inversely with concentration of drug. For example, with LSD-25, the first rates, after a functional heart was present in test embryos, were: control, 122 beats/minute; 100 μg./ml. LSD-25, 80/minute; 50 μg./ml. LSD-25, 97/minute; 10 μg./ml. LSD-25, 115/minute; 1μg./ml. LSD-25, 140/minute. Mean heart rates for all days up to hatching were: control, 147 beats/minute; 79, 93, 115 and 141 beats/minute for the 100, 50, 10 and 1 μg./ml. concentrations of drug, respectively. LSD data plotted as logarithm of dose against heart rate form a straight line. Using the least squares method, the resulting prediction equation is: heart rate equals 144 minus 32 times logarithm of dose. With yohimbine, mean atrial heart rates for 100, 50, 10 and 1 μg./ml. concentrations were, respectively, 102, 122, 131 and 152 beats/minute; control, 147. In yohimbine concentrations above 10 μg./ml., frequent atrioventricular dissociation, with a ratio of one ventricular beat to from 2 to 8 atrial beats, occurred. UML-491 had little effect on pulse rate. The effect of LSD-25 and UML-491 on embryonic heart rate apparently is unconnected with their antiserotonin activity: UML-491 has an antiserotonin activity over four times greater than that of LSD-25. Serotonin itself had no effect on pulse rate. The Sandoz Pharmaceutical Company kindly supplied the LSD-25 and UML-491.

Supported by National Institute of Mental Health grant MY-3235.

Some morphological effects of lysergic acid diethylamide and related agents on early embryonic development in Fundulus. JOSEPH A. BURKE.

To ascertain whether representative psychotropic drugs have an effect on development, fertilized eggs of *Fundulus* were exposed at 2- to 4-cell stage (Oppenheimer, stages 3 and 4) to 100, 50, 10 and 1 μg./ml. sea water solutions of lysergic acid diethylamide (LSD-25), methysergide (UML-491 Sandoz), yohimbine and serotonin. Twenty-five eggs per 50 ml. solution were grown in finger bowls at 21° C. The concentrations were low enough to obviate changes in osmotic pressure or pH. No inhibition was noted in stages from Oppenheimer 4 to 16: up to the stage when the forebrain expands to form the optic vesicle. At this stage all four substances exerted some degree of growth inhibition. LSD-25 was the most potent. In embryos in the 100 μg./ml. LSD-25 solution, heart formation was delayed for about 48 hours. No melanophores or xanthophores were visible; optic vesicles were small and often solid. The embryo was undersized and underdeveloped. The same effects were evident but to a lesser degree in the 50, 10 and 1 μg./ml. solutions. Hatching was inhibited in the higher concentrations; the embryos finally died if not removed from the LSD-25 solutions. In the UML-491 test embryos, there was similiar inhibition but a lesser degree: the embryos were smaller than controls; there were fewer melanophores and those were dispersed. Eventually the embryos hatched with controls, but such embryos remained slightly undersized. Heart formation was delayed about 24 hours. Serotonin in the concentrations used showed slight inhibitory action. Heart formation was not delayed; embryos in general were smaller. Yohimbine concentrations above 10 μg./ml. induced abnormal heart formation. Embryos in this substance, if hatched, soon died. Rated in order of potency for inhibition: LSD-25, yohimbine, UML-491 and serotonin. The Sandoz Pharmaceutical Company kindly supplied the LSD-25 and UML-491.

Supported by NIH grant MY-3235.

Dactyl chemoreceptors of brachyurans. J. CASE, G. F. GWILLIAM AND F. HANSON.

The presence of chemoreceptors sensitive to amino acids is demonstrated on all limbs of *Libinia emarginata, Callinectes sapidus* and *Carcinides macnas* by recording from small bundles dissected from the leg nerves during application of localized stimuli to various parts of the limb. These receptors, whose end organs are not known with certainty, respond well to boiled or dialyzed-aqueous extract of Mytilus, are but slightly stimulated by 25% and not at all by 200% sea water, and are unaffected by dilute hydrochloric and acetic acids. Methyl, ethyl, n-butyl and n-propyl alcohols at concentrations as high as 0.2 *M* are non-stimulatory, as are maltose at 0.5 *M* and lactose at 0.25 *M*. The polypeptides glutathione and tryptamine are non-stimulatory at 0.1 *M*. All amino acids tested were stimulatory, but of these dl-methionine, l-glycine, l-arginine, β-alanine, l-proline and l-lysine were active only at concentrations of 0.2 *M* or greater. The most active substance tested was l-glutamic acid with a response threshold approximating 0.001 *M*, either unbuffered or at pH 7.8, approximately that of sea water. Considerable specificity of the glutamic acid response is suggested by the poor stimulatory action of α-methyl glutamic acid and glutamine. Behavioral experiments in which these substances were applied to mouthparts and legs of *Carcinides* confirm these observations. An active compound applied to the mouthparts produces feeding movements or, if applied to a cheliped, causes immediate feeding movements followed by touching of the cheliped to the mouth.

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Secretory structures in the tube foot of starfish. A. B. CHAET AND D. E. PHILPOTT.

The means by which starfish manage to climb up the vertical side of a glass aquarium is usually explained by the suction cup theory. However, if tube feet adhered to a glass wall are cut, they will continue to remain in place even though the vacuum has been released. It has also been observed that the tube feet leave behind a stainable substance, as well as particles which can be observed under the electron microscope. With these preliminary observations in mind an attempt was made to study the ultra structure of starfish tube feet.

Tube feet of *Asterias forbesi* were obtained by allowing them to adhere to a glass surface, and then cutting the foot at its proximal end. They were fixed in osmic acid sea water and, after dehydration, embedded in methacrylate or vestopal. Observing both longitudinal and cross-sections in the electron microscope disclosed that the side of the tube foot, which contains at least two types of secretory glands and is covered with micro villi, is completely different from the base of the tube foot—the area which adheres to surfaces. The base of the tube foot contains many elongated tubes, each one possessing dozens of ellipsoidal structures appearing to be "secretory packets." Each "secretory packet," about $\frac{1}{2} \times \frac{3}{4}$ microns in size, is surrounded by a layer of fine granules, inside of which may be found 50 or so fibers (200 Å diameter), arranged much like the continuous fibers of mitotic spindles. It has been shown that the "secretory packets" are secreted through the walking surface of the tube foot. It is possible that they function in "gluing" the tube foot to either rough or smooth surfaces, thus aiding in locomotion. Identical structures have been found in *Asterias vulgaris*.

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Recovery of uricase activity in concentrated urea solutions. AURIN M. CHASE AND JEAN M. BUSSARD.

The oxidation of uric acid by uricase is inhibited in the presence of urea (Chase, 1957, *Biol. Bull.*, **113**: 320), although the experimental results can be greatly influenced by the kind and concentration of buffer used to stabilize the pH (Chase, 1956, *Biol. Bull.*, **111**: 299).

In addition to this immediate effect of urea, which (in the case of lower concentrations) is reversible upon dilution, a slower—relatively irreversible—inactivation of the enzyme (Worthington purified uricase) can also be demonstrated. This was observed when the enzyme was allowed to stand in 3 to 8 *M* urea solutions in 0.1 *M*, pH 9 borate buffer. After the uricase had been exposed to the experimental urea solutions for the desired time, samples were diluted ten-fold with buffer containing uric acid, and the initial rate of decrease of absorbance of the reaction mixture was measured at 290 mμ. The temperature of all solutions and of the spectrophotometer's cell compartment was maintained at 26° C. The rate of irreversible inactivation of the uricase was greater, the higher the concentration of urea to which the enzyme was exposed before dilution.

In addition to the reversible and irreversible inactivations just described, another effect of urea upon the enzyme was observed. At the highest urea concentrations studied (6 to 8 *M*), the uricase activity—as measured after the ten-fold dilution—increased rapidly during the first fifteen minutes or so in the undiluted urea solutions, and then decreased again slowly. This initial recovery of activity was relatively slight in 6 *M* urea solution but very pronounced in 7.5 or 8 *M* concentrations.

So far, such experiments have been performed only at 26° C., using borate buffer of pH 9. The effects of temperature, of pH, and of other buffers should obviously be investigated.

Aided in part by a National Science Foundation grant.

Effect of non-static conditions during gamete irradiation on Arbacia fertilization and injury. RALPH HOLT CHENEY AND CARL CASKEY SPEIDEL.

Fertilization membrane characteristics and injury expressed by developmental stages are determined *to a degree* by the state of motion during gamete irradiation. Gametes were exposed to 2537 Å ultraviolet (UV) rays at a constant distance while in a static state or while agitated by two-per-second oscillation excursions over one-half inch right and left from the stationary center. Shaken or non-shaken eggs after irradiation (4 seconds to 16 minutes) were inseminated with normal sperm. Membrane characteristics, developmental injury, and death were noted comparatively over a three-day period.

Four-minute UV-rayed shaken eggs produced concentric tight fertilization membranes. Non-shaken UV-rayed eggs, as a result of differential cytoplasmic damage, developed eccentric membranes. Such membranes usually assumed normal form some time prior to first cleavage. Normal eggs, fertilized by shaken or non-shaken sperm, showed no membrane eccentricity. Later abnormal stages were similar whether derived from irradiated sperm or eggs. Cultures

from irradiated shaken eggs exhibited earlier and greater percentages of death than cultures from irradiated non-shaken eggs. This greater damage under non-static conditions probably was associated with the fact that injury depended upon depth of nucleus from the surface during irradiation. Shaking assured more uniform damage by bringing more egg nuclei into position for maximal reception of the UV rays.

Shaken or non-shaken x-rayed (2 to 120 kr) eggs did not give rise typically to eccentric membranes, but tight membranes resulted from strong doses. Deep x-ray penetration caused equivalent effects regardless of motion, whereas shallow UV-ray penetration induced differential effects.

Significant sequences of fertilization membrane formation and adjustments were recorded by time-lapse cinephotomicrography.

This investigation was supported by a research grant (PHS RG-4326 C3) to C. C. S. from the National Institutes of Health, Public Health Service.

Monoauxenic culture of Arcella vulgaris. ANNA CICAK AND J. B. WITTENBERG.

At the turn of the century Engelmann captured a few individual protozoans which formed indubitable gas bubbles in their cytoplasm. Bles, 20 years later, made an extended study of gas bubble formation in one of these forms, *Arcella vulgaris.* The nature of the gas is not known. In order to carry further the study of gas secretion by the organism, viable, densely populated cultures are essential. Although Arcella is commonly maintained in infusion cultures, the population density is low and the majority of the animals are in an unsatisfactory physiological state. We have found that Arcella maintained in a simple salt solution will reproduce vigorously when fed a suspension of washed *Aerobacter aerogenes* every few days. The Arcella remain attached to the bottom of the Pyrex dish. Spent medium is poured off and replaced once a week. The cultures have not as yet been obtained free of other organisms. However, at this stage the cultures contain a very dense population of vigorous individuals, many of which are seen to be dividing at any one time.

Development of the Ilyanassa embryo after removal of the mesentoblast cell. ANTHONY C. CLEMENT.

The mesentoblast cell (4d) of the molluscan egg gives rise to the primary mesoblast bands and a portion of the entoderm. Within a period of about an hour after its appearance, the 4d cell of the Ilyanassa egg can be experimentally removed by puncturing or tearing it with a glass needle; following injury the cell swells and separates from the embryo, without damage to neighboring cells. The development of about a dozen embryos of *Ilyanassa obsoleta* has been followed after removal of the 4d cell. In the better cases, normal or nearly normal development has been observed of velum, eyes, cerebral ganglia and commissure, foot, operculum, statocysts, stomodeum, esophagus, and shell. The larval retractor muscle was seen in one case. The shell may give the appearance of being empty, except for the mantle and occasional masses of yolk spherules. The esophagus leads into an entodermal complex which is situated in front of the shell, and in which components of the liver and stomach can be recognized. Neither intestine nor heart has been observed to develop after removal of the mesentoblast cell. Absence of the intestine accords with Conklin's conclusion that this structure arises (in Crepidula) from the entodermal derivative of 4d. The larval heart of prosobranchs has been described as an ectodermal vesicle which pulsates by means of mesodermal muscle cells. The present experiments suggest that the heart musculature is derived from the 4d cell.

Rectification in skate electroplaques and its abolition by barium ions. B. COHEN, M. V. L. BENNETT AND H. GRUNDFEST.

The electroplaques of *Raja erinacea* and *R. ocellata* exhibit a marked increase in conductance when they are depolarized by more than 10 mV. The conductance change tends to restore the membrane potential to about 5 mV positive with respect to the resting value. The change requires one to several msec. to develop. It decays over several hundred msec. after a brief initiating pulse, and it is graded, depending on stimulus strength and duration.

The conductance increase occurs predominantly in the uninnervated, caudal face. It is qualitatively similar to the "delayed rectification" in squid axons, which has been attributed to increased K^+-conductance, and is a mechanism which increases the external current flow that is produced by the discharge of the electrically inexcitable innervated membrane. Though it is potential-determined, *i.e.*, electrically excitable, delayed rectification is not regenerative. Presumably, it lacks the component of inward flux of positive charge which provides the regenerative property of spike-generating membrane.

On applying Ba^{++}, the rectification was eliminated with little change in resting resistance, or in the responses to ionophoretically applied acetylcholine. In addition, there was a small depolarization. The neurally evoked responses were elicited for some time after the delayed rectification was eliminated, but the presynaptic fibers soon became inexcitable, often passing through a stage of repetitive firing, as indicated by "spontaneous" depolarizing potentials in the electroplaques.

Thus, the electrically inexcitable membrane component which produces the neurally and chemically evoked depolarization of the electroplaque was not affected by Ba^{++}, while the electrically initiated processes that produced the delayed rectification were blocked. The effect on potential-determined processes may be related to the block of K^+-conductance by Ba^{++} and other agents in various electrically excitable cells (Grundfest, this issue).

Permeability of red blood cells to alloxan. S. J. Cooperstein, Dudley Watkins, Evelyn Halpern and Arnold Lazarow.

Previous studies on the comparative distribution of injected radioactive alloxan, urea, mannitol and inulin in the various tissues of the toadfish suggested that alloxan may not enter the cell; it is possible, therefore, that alloxan may exert its diabetogenic effect by acting on the beta cell membrane. These studies have been extended by determining the degree of lysis of washed red blood cells in alloxan solutions of varying tonicities.

Since alloxan rapidly decomposes at body temperature and pH, these studies were carried out at 0° and pH 7.0. It was shown that under these conditions the half-life of alloxan was over two hours. A stock solution of alloxan was neutralized to pH 7.0 by adding NaOH to give a final concentration of 0.308 *M* alloxan and 0.085 *M* sodium ion; the total tonicity was 0.393 *M*. The concentration of ionized alloxan is presumed to be 0.085 *M;* that of un-ionized alloxan would then be 0.223 *M*.

One hundred microliters of washed human red blood cells were added to 3 ml. of various dilutions of the stock alloxan solution. The degree of hemolysis was measured spectrophotometrically at 650 mμ. The results were compared with those obtained using various dilutions of NaCl and of phosphate buffer (Na_2HPO_4-KH_2PO_4, pH 7.0). With alloxan, 50% hemolysis was observed at a final tonicity of 0.118 *M;* the final alloxan concentration was 0.092 *M*. When NaCl was used 50% hemolysis was observed at a tonicity of 0.15 *M* (0.075 *M* NaCl). Phosphate buffer produced 50% hemolysis at a tonicity of 0.113 *M* (0.423 *M* phosphate). Thus, at equal tonicities alloxan and phosphate buffer produce a similar degree of hemolysis, suggesting that at 0° and pH 7.0 the red cell membrane is impermeable to alloxan.

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Secretory epithelium of the swim bladder in Fundulus. Eugene Copeland.

Preparations of the swimbladder epithelium from Fundulus were studied with the aid of the electron microscope. Earlier studies of Regaud preparations had revealed no obvious mitochondria under the light microscope. The electron microscope demonstrates fine filamentous mitochondria with tubular or bleb-like cristae. The nuclei show a characteristically deep infolding on one side. The free surface of the cells (gas facing) is smooth and uninterrupted. The basal surface (capillary facing) possesses deep infoldings similar to those described in the cells of the distal tubule of the kidney. Vesicles of varying density, frequently in rows, are associated with the folds. Within the double membranes of the folds, *i.e.*, exterior to the cell, can be seen density-free, vacuole-like spaces. These are assumed to contain oxygen mobilized by the bladder mechanism. Larger bubbles have been observed in the space between epithelial cells. Desmosomes connect the free ends of the cells at intervals but the impression

is gained that gas escapes through areas or slots not so guarded. Patches of Golgi structure are seen. They have no obvious orientation in reference to the nucleus and the rest of the cell. Granules of varying size and density (lipid bodies?) are also seen. Correlation of their size and density with positional relationship to other structures has not been completed. All endoplasmic reticulum observed to date has been of the smooth variety. A very few "granular" strands were seen but they may well have been artifactual.

Oxygen toxicity among arthropods; in particular, modification of respiratory behavior and oxidative metabolism. F. EUGENE CORRIDEN.

Organisms across the entire taxonomic spectrum are known to be vulnerable to hyperoxia. Sensitivity is a variable of not only organism character but developmental stage and tissue type. Symptomatology has been rather well described. Commonly, the syndrome manifested includes an attenuation in respiration, suggesting an impairment of aerobic metabolism.

The provisional hypothesis adopted is that oxygen toxicity is mediated via an impedance of tricarboxylic acid cycle activity.

Insect forms frequently exhibit a depression in oxygen consumption subsequent to exposure to high tensions of oxygen (100%). Insects are known to possess a functional TCA cycle. Investigation has revealed that among pupal specimens of *Tenebrio*, pyruvic acid content (whole body) varies reciprocally with depression of oxygen consumption; the correlation is quite dramatic. Similarly, among pupal specimens of *Anagasta* and *Prodenia*, depression of oxygen consumption is accompanied by an accumulation of pyruvic acid and furthermore by a concurrent depletion of alpha-ketoglutaric acid. Pupal specimens of *Musca*, which did not exhibit any appreciable disparity in oxygen consumption, failed to show any modification in keto acid content. Pupal specimens of *Habrobracon*, in spite of a considerable retardation in oxygen consumption, maintained extreme stability in keto acid content. Extensions of experimental design to a study of *Uca pugnax* demonstrated toxicologic response to be paralleled by a rise in pyruvic acid content (muscle).

The tentative conclusion is that oxygen causes a block in the TCA cycle between the level of pyruvate and alpha-ketoglutarate. The speculation is that, specifically, the pyruvic oxidase system is the site of inhibition since it has a number of essential components known to be labile to oxygen *in vitro*.

Observations on the feeding activity of the isopod, Idothea baltica (Pallas). ARMANDO A. DE LA CRUZ.

Observations both in the laboratory and in the field were made on food preference, feeding behavior, rate and amount of feeding in the isopod, *Idothea baltica* (Pallas). These observations demonstrated that *I. baltica* is an omnivore that feeds readily on both the *Fucus* that harbors it and on living and dead animals. Values obtained from several experiments showed that on the average, an individual consumed about one-fifth of its body weight in half an hour when feeding on *Fucus*. When preying on a member of its own species, it consumed approximately half of its body weight in four hours.

It was demonstrated that the animals fed preferentially on the filiform processes on the surface of the *Fucus* thallus and on the younger and more tender portions of the plant. When feeding on other crustaceans, including individuals of their own species, they attacked living animals of up to one-half their own length, and dead or molting animals of almost their own size. They grasped their prey with all their pereiopods and usually feeding was initiated on the soft ventral parts. Eventually, the prey, including the exoskeleton, was totally consumed.

This work was undertaken as part of the student training program of the Marine Ecology Course and was partially supported by an ICA-NEC grant (Project No. 92-66-012-1-90197) and sponsored by the U. S. Office of Education, Department of Health, Education and Welfare.

Inhibitory action of a tissue extract on regeneration of Tubularia. AL. D. DINGLE.

Inhibition of regeneration of *Tubularia* has been obtained using supernatants of mature hydranth tissue breis at concentrations variously reported as ⅓ to ½ hydranth/ml. and 1⅓ to

2⅔ hydranths/ml. Wet weight determinations gave values ranging from 3 to 22 mg./hydranth, indicating that further quantitative studies should be based on hydranth weight rather than number. Supernatants, prepared by homegenizing washed mature hydranths in filtered sea water containing 125 μg./ml. streptomycin, and centrifuging the brei for 15 minutes at 10,300 *g*, were found to be inhibitory at concentrations between 15 and 30 mg./ml. The inhibitor was not species-specific, did not alter the pH of sea water, and its action could be simulated by equivalent concentrations of stem tissue (minus perisarc which contributes 20–40% of the wet weight).

When freshly cut stems were placed in the inhibitor, cellular protrusions usually capped the open ends, and further development stopped. Stems inhibited as long as 60 hours were capable of completely normal regeneration when returned to sea water. Application of the inhibitor to developing stems caused regression of any primordium already differentiated, and regenerated hydranths cytolyzed within 12 hours of treatment. Degeneration proceeded to the level of the neck in hydranths, and to the proximal end of the primordium in differentiating stems, then stopped—the stem did not cytolyze and remained capable of regeneration.

If the inhibitory supernatant is applied to a 2-mm. block of coenosarc left in the middle of a 10-mm. perisarc, the cells spread to fill the stem. This indicates that the extract does not act by stopping the migration of cells, and thereby preventing the build-up of a cell density critical for regeneration. It also demonstrates that the inhibitor does not act simply as a non-specific poison since the cells are able to undergo their normal movements.

Permeability studies on ground hog red blood cells. R. G. Faust and A. K. Parpart.

The relative rates of penetration of various monosaccharides and polyhydric alcohols into the ground hog red cell were studied by means of a photoelectric densimeter. Isosmotic concentrations (0.3 *M*) of these compounds were prepared in an isotonic NaCl-phosphate buffer which was adjusted to a desired pH. At pH 7 and 37° C., the rates of penetration to one-half diffusion equilibrium of the completely mutarotated sugars were as follows: for the pentoses; D-ribose 9 seconds, D-lyxose 16 seconds, D-xylose 22 seconds, L-xylose 24 seconds, D-arabinose 24 seconds, L-arabinose 29 seconds; for the hexoses: L-sorbose 33 seconds, D-galactose 57 seconds, D-fructose 125 seconds, and D-glucose 160 seconds. The disaccharide, sucrose, did not penetrate. There was no appreciable difference in the rates of penetration of the α- and β-isomers of D-glucose. Also at pH 7 and 37° C., the rates of penetration of the polyhydric alcohols are approximately linearly related to the number of carbon atoms in their molecule through 5 carbons. The rates were as follows: glycerol 1 second, i-erythritol 2.1 seconds, D-arabitol 3.5 seconds, L-arabitol 3.5 seconds, D-xylitol 3.5 seconds, ribitol 4.0 seconds, sorbitol 6 seconds, galactitol 9 seconds, and D-mannitol 11.5 seconds. The cyclic polyhydric alcohol, i-inositol, which contains 6 carbon atoms in its molecule, did not penetrate. The temperature coefficients (Q_{10}) which were calculated from the rates of penetration at 37°C. and 27° C. (at pH 7) were between 2.0 and 2.8 for all the sugars and between 1.2 and 1.6 for all the polyhydric alcohols that were tested. However, the influence of pH on the rates of penetration of the monosaccharides and the polyhydric alcohols was similar. Although pH variation affected their rates of penetration slightly, it was obvious that these compounds penetrated more slowly as the pH of their medium became more acid. Their rates of penetration were more rapid at pH 8 than at pH 6. This pH effect was reversible.

Comparison of the oxidation of C-1 and C-6 labeled glucose by islet tissue. James B. Field and Arnold Lazarow.

The amounts of $C^{14}O_2$ produced from glucose labeled in the one and six positions, respectively, were measured; a ratio greater than one suggests the presence of the hexose monophosphate shunt. Islet, liver and heart were removed from toadfish and goosefish; these tissues were incubated with radioactive glucose at 22° C. for two hours. The carbon dioxide produced was collected in hyamine and the radioactivity was measured in a liquid scintillation counter.

The amounts of glucose oxidized to $C^{14}O_2$ by islet tissue were 3- to 10-fold greater than that observed for liver and heart. With islet tissue the amount of $C^{14}O_2$ produced from glucose

labeled in the one position was several-fold greater than that produced from glucose labeled in the six position; this indicates the presence of the hexose monophosphate shunt in islet tissue.

Preliminary experiments were carried out in which varying amounts of either C-1 or C-6 labeled glucose were added to the islet tissue; the final glucose concentrations were 12.5, 25, 50, 100 and 200 mg. per 100 cc., respectively. The amount of C-6 labeled glucose oxidized by goosefish islet tissue increased progressively with increasing glucose concentration in the media; a maximal value was not reached at the highest glucose concentration (200 mg. per 100 cc.). By contrast, the amount of C-1 labeled glucose oxidized by islet tissue reached a maximum value at a glucose concentration between 25 and 50 mg. per 100 cc. At all glucose concentrations the ratio of CO_2 produced from C-1 vs. C-6 labeled glucose was greater than 2.0; at the lowest glucose levels, it was greater than 5.0. With liver the amounts of both C-1 and C-6 labeled glucose oxidized increased progressively with increasing glucose concentrations.

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Metachromatic granules in eggs of Hydroides. M. FILOSA.

Metachromatic granules in the cytoplasm of a number of marine eggs have been reported (Rebhun, Pasteels) to show specific movements during cleavage, in that they follow the centrioles. When unfertilized eggs of *Hydroides* are stained with toluidine blue (1:125,000) for five minutes, pink granules are found at random in the cortex of the egg. At no time during first or second cleavage of this egg do these metachromatic particles become associated with the mitotic spindle, but rather remain in the surface of the egg throughout. Eggs stained at first cleavage show the same localization of metachromatic granules as do eggs at the same stage that have been stained before fertilization. Eggs that were stained before fertilization were centrifuged at 8550 × g for both one and three minutes. When the germinal vesicle is intact, the metachromatic granules become localized near its centrifugal end. After the germinal vesicle has broken down, the location of the granules, up until first cleavage, differs, depending on the batch of eggs. In most cases the granules are moved internally and are located in the clear layer just below the lipid layer, with some granules scattered about in the clear layer. In some instances the granules form a layer in the yolk at the border of the clear layer.

Under oil immersion the metachromatic granules in these centrifuged eggs are seen to consist of two or three intensely stained small spheres on a light pink larger sphere. When unstained eggs were centrifuged at any stage between germinal vesicle breakdown and first cleavage, and then stained for five minutes, the metachromatic granules are always seen to lie in the clear layer just below the lipid cap. In these cases the particles are smaller and less intense in color than those of eggs that were stained before fertilization and centrifugation.

The influences of light and endocrines on the chromatophores of the mud shrimp, Upogebia affinis. MILTON FINGERMAN, R. NAGABHUSHANAM AND LORALEE PHILPOTT.

The anomuran *Upogebia affinis* possesses red chromatophores. Those on the telson and uropods can be readily observed and staged. Intact specimens on black and white backgrounds were placed under a series of light intensities ranging from 2 to 560 ft.c. No response to shade of backgound was observed. However, a response to intensity of incident illumination was apparent. The red pigment of specimens under an illumination of 2 ft.c. was maximally dispersed. With increased illumination (560 ft.c.) a slight decrease in the degree of pigment dispersion to stage 4 of the Hogben and Slome scale was noted in specimens on both backgrounds. To determine whether *Upogebia* displays a rhythm of color change, specimens were kept in darkness and in black pans under a series of light intensities ranging from 2 to 560 ft.c., and observed during the daytime and about midnight. No cycle was evident. However, the response to intensity of incident illumination was apparent at night as well as during the day. The red pigment was maximally dispersed in eyestalkless individuals and in intact specimens in darkness. The sinus gland of *Upogebia* lies on the dorsal surface of the supraesophageal

ganglia rather than in the eyestalk. Extracts were prepared of the eyestalks, sinus glands, and supraesophageal ganglia, and injected into eyestalkless *Upogebia*. In all cases concentration of red pigment was observed. The least effect was produced by the eyestalk extracts. The responses to the extracts of sinus glands and supraesophageal ganglia were large and approximately equal.

This investigation was supported in part by Grant No. B-838 from the National Institutes of Health.

The responses of the melanophores of eyestalkless specimens of Sesarma reticulatum to illumination and endocrines. MILTON FINGERMAN, R. NAGABHUSHANAM AND LORALEE PHILPOTT.

Eyestalkless specimens of *Sesarma* were exposed for two hours to a series of incident light intensities ranging from 2 to 1110 ft.c. The degree of melanin dispersion in these eyestalkless crabs was a function of the incident light intensity. The melanin was maximally concentrated at 2 and 20 ft.c. With increased light intensity the melanin began to disperse. This pigment was almost maximally dispersed in specimens exposed to an illumination of 1110 ft.c. Melanophores on isolated legs showed a similar direct response to incident light, although the amplitude of the response was not as great as was observed when the legs were attached to the body. To determine the role of endocrines in pigment migration in *Sesarma*, extracts were prepared of the sinus gland, optic ganglia, supraesophageal ganglia, circumesophageal connectives, and thoracic nerve cord. These extracts were injected into eyestalkless *Sesarma* whose melanin was maximally concentrated, and into specimens maintained under a high intensity of illumination so that the melanin was in a state intermediate between the fully dispersed and fully concentrated conditions. Each extract dispersed melanin. Extracts of sinus glands produced the least dispersion. In the case of the sinus glands, after the dispersing action of the extract, pigment concentration was noticed in the specimens whose pigment had been in an intermediate state at the time of injection of the extracts. Although the amount of melanin was not large, it was statistically highly significant.

This investigation was supported in part by Grant No. B-838 from the National Institutes of Health.

Responses of the melanophores of the grapsoid crab Sesarma reticulatum to light and temperature. MILTON FINGERMAN, R. NAGABHUSHANAM AND LORALEE PHILPOTT.

Sesarma exhibits a cycle of color change whose frequency closely approximates 24 hours. Crabs were placed in darkness and observed daily at 8–8:30 A.M., noon–1 P.M., 5 P.M., and 12 P.M. for one week. The pigment was less dispersed at midnight than at other times of day or night. Crabs were then tested to determine whether differences in incident light intensity and in shade of background would have an influence on the degree of melanin dispersion. During the daytime the melanin was maximally dispersed in animals kept in black pans under a series of light intensities from 2 to 1110 ft.c. At 2 ft.c. the melanin of animals in white pans was in an intermediate stage of dispersion. With increase in intensity of incident illumination a smoothly graded response was noted; the melanin became maximally dispersed in specimens in white pans under an illumination of 1110 ft.c. The responses to black and to white backgrounds and to incident light intensities between 2 and 1110 ft.c. were then determined between 11 P.M. and 3 A.M. The melanin of crabs in white pans was more concentrated than that of crabs in black pans at all light intensities. A response to intensity of incident illumination was also apparent; the melanin was more dispersed in crabs under the high intensities than under the lower ones, whether the crabs were in black or in white containers. However, at night the melanin did not disperse maximally in crabs on a black or a white background under any of the light intensities used. Responses to temperature were determined during the daytime with crabs in white pans under an illumination of 2 ft.c. At 3° C. the melanin was more dispersed than at 36° C.

This investigation was supported in part by Grant No. B-838 from the National Institutes of Health.

Effects of Blepharisma pigment on marine invertebrate development. B. A. Fong and H. I. Hirshfield.

The fresh-water ciliate, *Blepharisma undulans*, contains a red pigment that is photolytic and cytotoxic to a variety of cells and organisms. Initial studies were made on the effects of the pigment on gametes and developmental stages of the marine annelid, *Chaetopterus pergamentaceus*, and the sea urchin, *Arbacia punctulata*. These two species were selected since they are members of two major superphyla. Accordingly, differential responses to the pigment of these two species may have possible evolutionary significance.

The alcohol-extracted pigment was dried and a 1 mg./ml. concentration was prepared. This concentration and serial dilutions in sea water of 0.1, 0.01 and 0.001 mg./ml. were used.

The effects of the pigment in an intense light field and in the dark were noted on the gametes and developmental stages of the two species. A significant decrease in egg fertility was obtained, whereas the inseminated eggs showed less developmental delay and cytolysis. Exposure to light enhanced the cytolytic damage considerably.

Chaetopterus eggs exposed to the maximal concentration of pigment for 90 minutes, prior to insemination with untreated sperm, showed large numbers of abnormal and unhatched blastulae. In striking contrast, *Arbacia* showed abnormal fertilization membranes and 99% mortality. Those eggs of *Arbacia* that survived showed either abnormal cleavage patterns or developed normally through blastulation and hatching. Large numbers of abnormal larvae of both species were seen in the pigment-exposed groups.

Light and dark controls were kept for each series.

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Transplantation of distal limb tissues to upper arm stumps in adult Triturus viridescens. Richard W. Glade.

Pertaining to the problem of the establishment of pattern during limb regeneration, the following experimental series were performed: (1) implantation of three ulnae per stump (9 cases); (2) implantation of the phalanges of three digits per stump (12 cases); and (3) transplantation of a sheath of forearm skin to encircle the stump (10 cases). The bone transplants carried some adherent connective tissue and muscle. The skin grafts, however, rarely included muscle, and then only as an occasional fiber. Two control series involved the implantation of the distal halves of two humeri (15 cases), and the implantation of the proximal halves of two mandibles (11 cases) per stump. In all series, mock operations were performed on the opposite forelimbs, which served as controls.

Regeneration of the experimental stump was slightly delayed, beginning with the blastema or cone stage in the ulna, phalanges, and mandible series (6/9, 8/12 and 11/11 cases, respectively) as compared with its control. Delay occurred in 7/15 cases in the series in which humeri were implanted. In all of the remaining animals, the experimentals and controls regenerated at equal rates. The forearm skin series differed from the above in that in only 2/10 cases were the experimental regenerates delayed, while in 3/10 cases the controls were delayed. In 5/10 of the cases regeneration of the two limbs occurred at an equal rate.

Concerning the morphology of the regenerates, a variable degree of shortening in the length of the forearm, without significant inhibition of the hand, was observed in the ulna, phalanges and skin series. In no animal was the control forearm regenerate shorter than the experimental. An histological examination of the regenerates is being carried out.

This work was done with the technical assistance of Miss Nancy J. Scott.

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Movements of ^{24}Na and ^{42}K in the squid retina. W. A. Hagins and R. G. Adams.

Exchange of ^{24}Na and ^{42}K with sodium and potassium in the isolated squid retina has been measured. The retinas were isolated in darkness under an infra-red image converter and mounted in a thin chamber between two rapidly flowing layers of artificial sea water. Isotope solutions were flowed past the retina and then washed away with non-radioactive ASW and

the activity remaining in the tissue monitored with a counter placed against the chamber. The responses of the retina to light were measured with electrodes fixed in the chamber. ^{24}Na moved into 30% of the retinal water within one minute and could be washed out with similar speed. Long exposure to ^{24}Na revealed an additional slow uptake into a further 10% of the retinal water in two hours. This latter compartment lost its ^{24}Na slowly with an exponential time course whose half-time was 30 minutes at 10° C. ^{42}K uptake by the retina was slow and roughly exponential, with 70% of the intracellular K^+ exchanged in 3 hours. ^{42}K outflux was also slow and precisely exponential. It was not affected by raising the external K^+ from 10 to 30 mM even though the retinal photocurrent was reduced 30%. Similarly, 2 mM procaine left the outflux unaffected while increasing the photocurrent by 60%. In procaine-ASW, light which caused a total flow of 0.02 micromole of ions per $cm.^2$ of retina produced no change in the ^{42}K outflux, though an increased total outflux of this size would have been just detectable. In the absence of procaine, light did produce a small increase in ^{42}K outflux lasting for 10 minutes after cessation of illumination. Light adaptation which reduced the retinal sensitivity 30-fold left the fluxes unaltered and the K content of the cells essentially undisturbed. Since the exposure used isomerized less than 1% of the retinal photopigment, light adaptation must involve changes other than simple destruction of rhodopsin or gross changes in the Na and K content of the receptors.

Light-induced current from the receptors of the squid retina. W. A. Hagins, R. G. Adams and H. G. Wagner.

Isolated, dark-adapted squid retinas at 10° C. bathed in flowing oxygenated artificial sea water (ASW) maintain their sensitivity to light for many hours. Characteristically, the retina responds to the onset of illumination with a transretinal voltage rising sigmoidally from nearly zero to a steady level. The value reached is directly proportional to light intensity between 10^6 and 10^{11} photons $cm.^{-2}$ $sec.^{-1}$ (500 mμ). When the light is turned off the voltage returns rapidly to zero. The rise and fall of the voltage show a latency of about 80 msec. and a transition time of about 100 msec.; the exact values depend on temperature. Since the electrical impedance of the retina is essentially an ohmic resistance of 20–30 ohm cm. between 5 and 5000 $sec.^{-1}$, a light-induced transretinal voltage implies the flow of current within the retina. With the two surfaces of the retina short-circuited through a feedback amplifier, measurement of the current shows it is of the order of 10^6 electronic charges per incident photon (500 mμ). The origin of the photocurrent is attributed to the photoreceptors since (1) these cells are the bulk of the retina and the principal nervous elements, and (2) the action spectrum of the photovoltage closely resembles the absorption spectrum of squid rhodopsin, and (3) the amplitude of the photovoltage varies with depth as recorded by micro-electrode, consistent with the presence of a positive current arising in the region of the receptor cell bodies, flowing longitudinally between them and ending near their photo-pigment-bearing ends. The electro-chemical nature of the current source has been studied by ionic substitution in the solutions bathing the retina. Replacement of Na^+ by $choline^+$ reversibly reduces the photocurrent by 30%, as does increasing the external $[K^+]$. Conversely, removal of K^+ from ASW increased the current slightly while 2.0 mM procaine increased it 50%. It is thus suggested that the photocurrent depends in some way on the membrane potential of the receptors but not on the presence of electrical excitability in the receptor axons.

The effect of polyvinylpyrrolidone on volume of the isolated rat lens. Clifford V. Harding.

When isolated rat lenses are maintained in Ringer-Locke solution at low temperature, they slowly increase in volume and lose transparency. It has been suggested that the increase in volume is due to the colloid osmotic pressure of the lens, which would become effective following the gradual inactivation of ion transport mechanisms under the *in vitro* conditions employed. Other explanations for the swelling are possible. This suggests, however, that lenses maintained in a solution of proper colloid osmotic pressure would not undergo an increase in volume. The earlier work of Pau with both lens and cornea demonstrated this to be the case. In the present studies, rat lenses were maintained at approximately 5° C. in Ringer-

Locke containing various concentrations of polyvinylpyrrolidone (PVP), a synthetic polymer which can be obtained at different molecular weights. The two molecular weights used were approximately 40,000 (K-30) and 360,000 (K-90). It was found possible to control lens volume by adjusting the concentration of PVP in the medium. For example, in low concentrations of K-30, the lens swells; in high concentrations, it shrinks, and at 15%, approximately normal volume is maintained and transparency is not lost over long periods of time (48 hours and longer). Regardless of the mechanism whereby PVP controls the volume of the lens, it is suggestive that a proper adjustment of the colloid osmotic pressure of the medium can prevent the swelling which normally follows lens isolation. This could conceivably avoid damage to cells and fibers, which might accompany lens swelling, and thus maintain the lens in a viable state *in vitro* for longer periods of time.

It is planned to check this by measuring the extent of cellular proliferation in the lens epithelium, a factor which is apparently a sensitive index of the viability of the lens.

Colloid osmotic pressure might also be the effective characteristic of solutions of PVP which have been shown to prolong the time that rabbit corneas can be maintained *in vitro* without losing their capacity for transplantation (La Tessa).

Thymidine incorporation in epithelium of fish lens maintained in vitro. CLIFFORD V. HARDING AND B. D. SRINIVASAN.

Tritium has been localized autoradiographically in whole-mount preparations of lens epithelium, a single layer of cells, from the rabbit and rat. The use of whole-mounts for this purpose makes possible a comparison of the extent of incorporation of tritium-labelled compounds in one portion of the epithelium with any other portion, in one preparation. This procedure has proven useful in studies on the incorporation of tritium-labelled thymidine in normal and injured epithelium.

It was thought of interest to extend this study to the lenses of cold-blooded animals. These would have the advantage of enabling a study of temperature effects in the intact animal under physiological conditions; and, they would also, perhaps, furnish a more favorable material for *in vitro* studies on cell division. Results of the present investigation have shown that the whole-mount method can be applied to the lens epithelium of dogfish, tautog, and sea bass. Freshly isolated dogfish lenses were incubated for two hours in a solution of elasmobranch Ringer (made up in glass-distilled water, pH approximately 7.5) plus tritium-labelled thymidine at 2.5 to 5 μc/ml. (Spec. act. 1.9 curies/mM), after which they were fixed in 3 parts absolute alcohol: 1 part glacial acetic acid. Whole-mounts were then prepared for autoradiography. The autoradiograms were exposed for 7 to 17 days before photographic development. The results show incorporation by many of the epithelial cells. The cells showing incorporation are relatively more scattered than in the rabbit lens, where the germinative zone is relatively localized.

Experiments were also carried out in which dogfish lenses were incubated in elasmobranch Ringer (with dextrose) for various periods of time before exposure to thymidine. Lenses maintained in elasmobranch Ringer at 21° C., for at least up to 22 hours before a two-hour exposure to tritium-labelled thymidine, showed incorporation of thymidine. Similar results were obtained with the epithelium of tautog lenses, maintained in teleost Ringer at 16° C. for 22 hours. The results suggest the possible use of this tissue for *in vitro* studies on cell division.

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Stimulation of Arbacia sperm respiration by egg substances. RALPH R. HATHAWAY.

Arbacia fertilizin is generally thought to depress the respiration of specific sperm. In a reinvestigation of this question, *Arbacia* sperm respiration and motility were markedly increased by a substance which freely diffuses from eggs. This *Arbacia* sperm-activating substance was prepared as the sea water supernatant of jellyless eggs. Such a sperm-activating preparation has no sperm-agglutinating properties, is diffusible in dialysis, soluble in alcohol, heat-stable, and non-volatile. The activator is evidenly a substance distinct from fertilizin.

When *Arbacia* sperm activator is mixed with an excess of specific sperm in glycyl-glycine buffered sea water, there is a four- to 20-fold increase in respiration rate. The high rate of O_2 consumption rapidly decreases as a power function of time. When the activator is in excess, the elevated respiration rate slowly decreases as a linear function of time. The pH is essentially constant throughout. When activator-sperm mixtures are centrifuged after one-half hour, the supernatants no longer contain activating properties. Conversely, sperm washed from the activator after one hour do not respond to fresh activator preparation. These facts suggest a mutual exhaustion of the activator and a sperm substance essential for sperm response to the activator.

Sperm activator occurs in *Arbacia* eggs from Woods Hole, Mass., and Alligator Point, Florida. Sperm activators were also found in *Lytechinus variegatus* and *Mellita quinquies-perforata* from Alligator Point. *Asterias forbesi* eggs from Woods Hole yielded no sperm activator. In specificity studies, *Arbacia* activator failed to stimulate sperm of *Lytechinus*, *Mellita* and *Asterias*. *Lytechinus* sperm activator did not affect *Arbacia* sperm, but *Mellita* activator caused a moderate increase in *Arbacia* sperm respiration.

Sperm of *Arbacia*, *Lytechinus* and *Melita* have invariably displayed an increased oxygen consumption in the presence of homologous fertilizin. This response may be due to the presence in fertilizin preparations of small quantities of active sperm activator substance(s).

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Egg jelly dispersal by Arbacia sperm extracts. RALPH R. HATHAWAY, LEONARD WARREN AND JOEL G. FLAKS.

Various substances, some containing sialic acid, are released by *Arbacia* sperm when they are treated with either *Arbacia* fertilizin or *ca.* 10^{-4} *M* sodium lauryl sulphate (see separate abstract). These sperm extracts affect *Arbacia* eggs by causing the disappearance of the egg jellies in 15 to 60 minutes at room temperature. Eggs which have lost their jelly coats are easily recognized by their close contact with each other. In addition, when *Arbacia* eggs are exposed to *Asterias* sperm, the egg jellies become impregnated with trapped non-fertilizing sperm. Subsequent exposure of these eggs to *Arbacia* sperm extracts results in the disappearance of the jelly coat and the release of the starfish sperm. This observation suggests that the disappearance of the egg jelly is caused by a true jelly dispersal rather than by a precipitation and contraction of the jelly, as is sometimes reported when other sperm extracts, *e.g.*, anti-fertilizin, are mixed with eggs. Furthermore, in preliminary tests supernatants from eggs treated with the sperm extracts contained more fucose than supernatants of control eggs. Since fucose is a major constituent of the egg jelly, this result indicates solubilization of the egg jelly coat.

The jelly "dispersing" activity is destroyed by heat (80° C. for 5 minutes), and acid (pH 3.0 for 5 minutes), and is inactive at 0°. These properties distinguish this agent from anti-fertilizin, and suggest an enzymatic activity.

The sodium lauryl sulfate extracts of sperm contain antifertilizin which precipitates a membrane around the jelly coat. Observation of the jelly "dispersing" activity of these preparations can be made only after the antifertilizin is removed. The jelly precipitating activity can usually be removed by centrifugation at 33,000 *g* for 35 minutes. The pellet contains the jelly precipitating activity and all of the sialic acid which was extracted from the sperm by the sodium lauryl sulfate. The 33,000 *g* supernatant contains a strong jelly coat "dispersing" activity.

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The pseudocilia of Tetraspora. WALTER R. HERNDON AND DELBERT E. PHILPOTT.

The immobile, paired, pseudocilia of the vegetative cells of the chlorophycean alga *Tetraspora lubrica* Roth strongly resemble the flagella of its motile cells when examined microscopically, especially with lower magnifications. However, with oil immersion and phase contrast, it is clear that these protoplasmic strands have a structure different from that of typical flagella; they are comprised of an axial portion surrounded by a relatively broad sheath. The axial

portion is of greater optical density than the sheath and is beaded; the sheath stains readily with methylene blue. Pseudocilia of colonies fixed in 1% osmic acid or 5% potassium permanganate, embedded in methacrylate and sectioned show the same structure as seen in the living material when examined with the light microscope, except there is in some a slight shrinkage of the sheath and in these the axial portion may be somewhat coiled. In sections thus prepared and examined with the electron microscope, the sheath and axis are clearly distinguished. The sheath is about one micron in diameter and relatively uniform along its length. The axial portion appears to be basically tubular but has numerous constrictions which delimit spherical and elongate segments of varying sizes (mostly 0.2–0.4 micron in diameter). At its insertion, the axial portion appears to be an extension of the anterior cytoplasm peripherally disposed about a vacuolate interior. The beaded structure of the pseudocilia was found in colonies of different ages, in collections from different localities, and in a different species of the genus. The position of the pseudocilia of vegetative cells and some features of their morphology suggest that they might be produced by a process which is a modification of that producing the typical flagellar structure of motile cells also examined with the electron microscope, for comparative purposes, in this study.

The stage at fertilization of the egg of Fundulus heteroclitus. CHARLES W. HUVER.

According to Costello, Davidson, Eggers, Fox and Henley (1957) in *Methods for Obtaining and Handling Marine Eggs and Embryos*, the stage at which the egg of *Fundulus heteroclitus* is fertilized has not been determined. This is due to the fact that the polar bodies of this popular embryological material have not been previously described.

While examining time-lapse movies of fertilization and cytoplasmic streaming during blastodisc formation, both polar bodies were observed, apparently for the first time. The polocytes have now been observed in a total of 15 eggs. They project into the perivitelline space from the rim of the funnel-shaped mouth of the micropyle. The first and second polar bodies are of equal size (0.04 mm.-diameter). They are in close contact with each other and are incompletely elevated from the egg surface.

The reasons why students of early *Fundulus* development have previously failed to observe the polar bodies are: (1) the mature egg is very difficult material for proper fixation and sectioning, (2) the extreme transparency of the polocytes, (3) the relatively small size of the polar bodies in relation to the size of the egg, (4) the resemblance of the polar bodies in size and in shape to cortical alveoli, and (5) the masking effect of the thick chorion which encloses the egg.

The first polar body becomes clearly visible upon the elevation of the chorion at approximately three minutes after insemination. The time of emergence of the first polar body is unknown. However, it appears to be present in freshly stripped unactivated eggs where it is closely compressed to the egg surface. The actual emergence of the second polar body can be seen in fertilized eggs. It arises next to its predecessor in a smooth but quick movement at 4.5–5.0 minutes after insemination. The presence of the first polocyte in the ripe unfertilized egg, and the rapid formation of the second after sperm entrance, indicate that the egg is a secondary oocyte when fertilized. The emergence of the second polar body so soon after insemination suggests that the second maturation division proceeds to an advanced stage before it reaches the pre-fertilization arrest.

N.I.H. Predoctoral Fellow.

Effects of temperature on charge transfer through a receptor membrane. NOBUSADA ISHIKO AND WERNER R. LOEWENSTEIN.

Mechanical stimulation of the nerve ending of Pacinian corpuscles produces transfer of charges through its receptor membrane. The energy requirements for the transfer are markedly influenced by temperature. For example, the mechanical stimulus strength necessary to produce a given generator potential at 25° C. may be reduced to one-third at 35° C. A temperature change alone, however, elicits no detectable transfer.

The main effect of a temperature change is to vary the rate of rise and the amplitude of the mechanically elicited generator potential. Both increase linearly with temperature, with

Q_{10} of 2.5 and 2.0, respectively (15° C.–35° C.). The primary event here appears to be the effect on the rate: the active phase is shorter than the rise time of the generator potential and, hence, the total charge transferred increases as the temperature rate augments with temperature. The rate of fall of the generator potential is not appreciably affected by temperature.

The first Ranvier node, the site of nerve impulse initiation, behaves quite differently. The amplitude of the nodal action potential remains practically unchanged between 20° and 40° C., and its duration increases considerably with temperature (Q_{10}, 3–4). The nodal threshold varies inversely with temperature; impulse initiation fails completely below 12° C., although the receptor membrane still produces generator potentials.

A simple model for receptor excitation is that of ions diffusing simply along their gradients through mechanically stretched pores of the receptor membrane. This model is now no longer tenable without modification. The high activation energy of the rate-limiting process in excitation, 16,300 cal./mole (as derived from the temperature dependence of the rate of rise of the generator potential), forces at least one additional element upon this or any other model, namely an energy barrier for charge transfer. To surmount the barrier, energy may be supplied directly by heat transfer, or indirectly by a chemical reaction.

Stimulation of conidia formation at the growing tips of Neurospora crassa by x-irradiation. J. KEOSIAN AND B. P. SONNENBLICK.

N. crassa grows with a sharp frontier in an agar minimal medium in growth tubes. When this frontier is x-irradiated (9000 r at 182 kvp) the following are noted:

1. Formation of a dense band of conidia, about 5 mm. wide, at the site of the original irradiated frontier, visible in 6–8 hours. This band persists as to location, width and relative density, even after the tube is overgrown with conidia.

2. The conidial band forms when the frontier alone is irradiated and the rest of the growth is lead-shielded, but the band does not form when the frontier is shielded and the remainder exposed. This suggests that the radiation influence is limited to the growing tips.

3. Age appears to have no significant effect on the formation of the conidial band since irradiation of 24-, 48- and 72-hour cultures produced comparable results.

4. The threshold for the formation of the band is between 1000 r and 2000 r, resulting in a definite but sparse band. With increasing dose the band becomes denser with conidia. Experiments performed with a previous machine (85 kvp) indicated an optimum effect at about 9000 r.

5. Retardation of growth rate for the first six hours, followed by an acceleration of growth rate over normal. This increase is small but is consistently found in the irradiated cultures.

6. Conidial band formation at the original frontier, with visibly different characteristics from that induced by radiation, occurs after exposure for given periods of time at certain temperatures (10° C. and 42° C.).

Histochemical and biochemical analyses are projected in an attempt to ascertain whether the localized conidial band formation described above is a generalized stress response to an unusual environment, or whether there are differences in the response to radiation *per se* and to other agents.

Observations on the submicroscopic cytology of the epithelial cells of the cardia of a dipteran insect, Hypoderma bovis. RICHARD G. KESSEL.

The cardia of dipterous insects is the most anterior portion of the midgut. It is known to be highly active in secretion and involved in the production of the peritrophic membrane. The lumen surface of the epithelial cells possesses a multitude of microvilli, often arranged in hexagonal array. Numerous blebs of the apical portion of the plasma membrane can be observed. A budding or blebbing of the distal portion of the microvilli is also apparent, but on a smaller scale. The most abundant cellular organelle is the endoplasmic reticulum. In the basal portion of the cells, the reticulum consists of numerous, parallel, interbranching, rough-surfaced lamellae. In the central portion of the cell, the lamellae of the endoplasmic reticulum are observed to be greatly expanded at their end region (forming ergastoplasmic sacs) or along their length. In the most apical portion of the cell, numerous smooth- and rough-surfaced

vesicles are characteristically present. The latter may also be observed within the large blebs of the apical plasma membrane. It appears that the secretory vesicles have their origin from the endoplasmic reticulum. Mitochondria and parallel, smooth-surfaced lamellae of the Golgi apparatus are also present, but both are extremely small. The Golgi material was never observed to be conspicuous. The cells abut upon a basement membrane approximately 0.75 micron thick. The nuclear membrane is porous and a prominent nucleolus is present. Observations at various stages in the formation of the peritrophic membrane suggest that the microvilli contribute, at least in part, to its formation. (Supported by a grant, RG-6942, from the National Institutes of Health, U.S.P.H.S.

An electron microscope study of the mitochondria-rich "chloride cells" from the gill filaments of fresh water- and sea water-adapted Fundulus heteroclitus. RICHARD G. KESSEL AND H. W. BEAMS.

The ultrastructure of the gill filaments of *Fundulus heteroclitus* was studied with special reference to the mitochondria-rich chloride cells which were earlier described with the light microscope by Copeland (1948, 1950). Filaments of animals from sea water were examined, as well as those from animals adapted to fresh water for twelve hours.

In sea water-adapted animals, the chloride cells appear very dense, due to the numerous, filamentous mitochondria. The cristae of the mitochondria are mainly oriented parallel to its long axis. Located among the mitochondria and closely associated with them is a prominent, tubular network of an agranular type. This structure is similar to a specialized form of endoplasmic reticulum described in the pseudobranch gland (Copeland, 1959). The nucleus is characterized by two areas of different electron densities, each of which appears lobated.

In fresh water-adapted animals, the chloride cells do not appear to be as densely populated with mitochondria. In addition, the mitochondria appear shorter and thicker than those in sea water and the cristae in many cases are oriented transversely. The network of smooth-surfaced, branching tubules (thought to represent a form of the endoplasmic reticulum) appears more abundant, but smaller in size, than is the case in sea water. A continuity of the tubular endoplasmic reticulum and the plasma membrane of the cells is observed under both sea water and fresh water conditions. A small amount of rough-surfaced lamellae of the endoplasmic reticulum is also present. The nucleus appears homogeneous, is of moderate electron density and often contains a nucleolus. (Supported by grants from the National Institutes of Health, U.S.P.H.S.)

The effect of waves in influencing the composition of a flora of attached sea-weeds. JOHN M. KINGSBURY.

The granite jetty projecting across the harbor entrance at West Falmouth, Mass., lies parallel to the general shore outline of Buzzards Bay. Its western, bay exposure (with 69% of total wind over open water fetch of 6¼ miles or more) receives constant wash of waves or swells, usually producing spray. Its eastern, harbor side (maximum fetch less than ½ mile, wind exposure about 2%) rarely experiences waves and never has significant spray. Tidal effects, water temperature, light, currents, and rock substrate were observed or measured and found identical or nearly so at the six collecting stations. Stations were paired, leeward and windward members opposite, the first pair 10 feet from the projecting end, second and third pairs each 12 feet further to landward. At each station all manually removable vegetation was collected from 6-inch quadrants forming a vertical transect from highest growth to jetty bottom.

A *Calothrix* zone, above lowest high water, was present only to windward. A 3-foot (vertical) zone of *Fucus vesiculosus*, present on both sides, was ½ to 1 foot lower on the leeward side. Its lower boundary leeward corresponded with extreme low tide level; windward it was bounded by mean low and lowest high tide levels. The leeward side showed fewer germlings, presence of bladders and larger plants in comparison with windward. A zone, between highest and lowest low tide levels, dominated by *Enteromorpha linza*, occurred only on the windward side. Of 35 species found on windward, 8 were not found on leeward; similarly, 2 of 29 leeward species were not found on windward. *Chondrus crispus*, the principal non-epiphytic member below the *Fucus* and *Enteromorpha* zones, was displaced upwards 6 to

9 inches on the leeward side. On the leeward side the vertical level equivalent to the windward *Enteromorpha* zone was occupied chiefly by the downward *Fucus* and upward *Chondrus* displacements.

Centrifugal influence on the electroretinogram of the frog. STEPHEN T. KITAI.

The existence of centrifugal fibers in the optic nerve was investigated by studying the effects of Nembutal on the electroretinogram b-wave responses of frogs under the following conditions: (1) spinal pithing, (2) spinal pithing and unilateral optic nerve sectioning before the injection of the Nembutal, and (3) section of the optic nerve after the Nembutal effect on the electroretinogram responses appeared.

The electroretinogram responses were recorded from the cornea of frogs, using sodium chloride-plated silver electrodes. The signals from the electrodes were fed into Grass P6 d.c. pre-amplifier and finally recorded by an oscilloscope. The electroretinogram records were taken before and after the Nembutal injection under spinal pithed condition, and under spinal pithed unilateral optic nerve-sectioned condition.

Changes in the magnitude of the electroretinogram responses due to extraneous variables, such as changes in the retinal area stimulated or in the amount of stimulus light, were controlled. Optic nerve sectioning did not damage the retinal artery. The electroretinogram responses from the unoperated contralateral eye were taken as a control.

The effect of Nembutal on frogs under spinal pithed condition was to decrease the electroretinogram responses. The Nembutal injection failed to decrease the electroretinogram responses when the optic nerve was sectioned before the injection. The decreased electroretinogram responses by Nembutal injection were eliminated when the optic nerve was sectioned.

Sectioning of the optic nerve abolished the effects of Nembutal on the electroretinogram b-wave responses. This is interpreted as an evidence for the existence of the centrifugal fibers in the optic nerve, since the higher center upon which the drug acts no longer connected with the retina.

Krebs cycle dehydrogenase systems in eggs of Asterias as measured with a tetrazolium salt. EVELYN KIVY-ROSENBERG, FRANCES RAY AND HELENE ELEFANT.

The quantitative study of substrate-dependent dehydrogenase activity has been in progress for several years (Kivy-Rosenberg, Cascarano and Merson, 1959; Biol. Bull. 117). Work was continued on *Asterias* uninseminated and inseminated eggs. An extensive series of substrates was utilized, including those which require DPN or TPN as cofactors and succinate which requires none. Included in this series were four which are involved in the Krebs cycle: alpha-ketoglutarate, and malate, each with DPN, isocitrate with TPN, and succinate. The tetrazolium salt used as a hydrogen acceptor was 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT).

Homogenates of uninseminated and inseminated eggs of the same female (of a large series of starfish) were incubated aerobically for one hour at 37.5° C. in media containing INT and substrate or appropriate controls. Formazan was extracted and the quantity of reduced tetrazolium measured photocolorimetrically. The substrate-dependent dehydrogenase activity was expressed as micrograms of formazan per milligram of protein.

Of the fifteen hydrogenase systems measured, three in the Krebs cycle are among the most active: alpha-ketoglutarate ranking first; isocitrate, second; malate, third, in practically all cases. Occasionally ranks of alpha-ketoglutarate and isocitrate were interchanged, and malate ranked fourth. The activity of the other member of the cycle, *i.e.*, succinic dehydrogenase, was among the lowest of the entire series. The ranks mentioned were similar for both uninseminated and inseminated eggs.

Insemination resulted, in general, in a rise of dehydrogenase activity in homogenates (as we had reported earlier also for *Spisula*). In a few cases the reverse was true. On the whole, the activity of the Krebs cycle dehydrogenases followed the general trend: *i.e.*, a rise (when there was a general rise with insemination), and a fall (when the reverse was true). This was true for the one which showed relatively low activity as well as the three highly active dehydrogenases.

Further consideration and study will be given to the surprising relationship between the low activity of succinic and high malic dehydrogenase systems.

The iron requirement of some marine plankton algae. DANA D. KRAMER AND JOHN H. RYTHER.

The iron requirement of some marine phytoplankton was investigated by determining final populations attained in media containing different concentrations of Fe-EDTA. The organisms studied were three oceanic species (*Coccolithus huxleyi, Chaetoceros* sp. and an unidentified centric diatom "13-1") and four inshore species (*Skeletonema costatum, Isochrysis galbana, Amphidinum carteri* and an unidentified flagellate "3C"), all maintained in pure culture at the Woods Hole Oceanographic Institution.

The algae were grown in offshore ocean water enriched with all essential nutrients except iron, until further growth was obviously iron-limited. Iron-deficient cells were then inoculated into media enriched respectively with 0, 2.6, 6.5, 13, 26, 65 and 130 μg. Fe/L. These cultures were incubated (1000 foot candles, 20° C.) until maximum growth was attained (*ca.* 7 days), after which population density was determined by cell counts.

Coccolithus could not be made iron-deficient. After several transfers, more growth occurred in media without than with iron added. All other species became iron-deficient after 2–3 transfers, with no growth in control cultures containing no added iron. The two other offshore species (*Chaetoceros* sp. and "13-1") reached maximum densities at 26 and 2.6 μg. Fe/L, respectively; growth was depressed at higher levels. Of the inshore species, *Isochrysis* and *Amphidinum* reached maximum growth at 65 μg. Fe/L; populations of *Skeletonema* and "3C" were still increasing at the highest iron concentration employed.

Skeletonema, an unbiquitous shallow-water diatom never found in the open sea, does not grow at iron concentrations supporting maximum growth of the oceanic species *Coccolithus* and "13-1." It is suggested that the higher level of iron in coastal waters associated with land drainage may influence or control the distribution of this and other neritic species. This work was carried out as part of the student training program of the Marine Ecology Course.

Effect of nicotinamide on pyridine nucleotide levels and cleavage rate of eggs of Spisula solidissima. STEPHEN M. KRANE AND ROBERT K. CRANE.

It was previously demonstrated in this laboratory that the major change in the concentration of pyridine nucleotides in early cleavage stages of marine eggs was a net increase in triphosphopyridine nucleotide in its reduced form (TPNH). In the present study, eggs of *Spisula solidissima* were incubated with nicotinamide in an attempt to modify the levels of pyridine nucleotides as well as the rate of cleavage. Pyridine nucleotides were measured fluorimetrically. The concentration of diphosphopyridine nucleotide (DPN) in unfertilized eggs incubated in nicotinamide (1×10^{-3} *M*) in sea water increased linearly from 210 millimicromoles/ml. cells to 535 millimicromoles/ml. cells in three hours. With lower concentrations of nicotinamide the increase was more striking (1010 millimicromoles/ml. at 1×10^{-4} *M* nicotinamide; 781 millimicromoles/ml. at 1×10^{-5} *M* nicotinamide). In eggs transferred to nicotinamide (1×10^{-2} *M*) in sea water 40–60 minutes after fertilization, the DPN levels were doubled, compared to controls three hours later, and cell division completely but reversibly blocked. Cleavage was blocked in concentrations as low as 5×10^{-4} *M* but only delayed at 1×10^{-4} *M*. Unfertilized eggs incubated three hours in nicotinamide (1×10^{-4} *M*) showed no change in TPNH levels compared to controls (5 versus 4 millimicromoles/ml.) despite marked increase in DPN (820 versus 194 millimicromoles/ml.). In fertilized eggs in nicotinamide, TPNH was 72 millimicromoles/ml. versus 34 millimicromoles/ml. in controls, whereas DPN was 1280 millimicromoles/ml. versus 185 millimicromoles/ml. in controls.

Effects of nicotinamide in *Spisula* eggs were therefore as follows: (1) Reversible inhibition of cell division; (2) Increase in levels of DPN in fertilized and unfertilized eggs; (3) Increase in TPNH *only* if cell division had proceeded.

Mechanisms of nerve impulse generation in a Lorenzinian ampulla. WERNER R. LOEWENSTEIN.

Single isolated ampullae of dogfish (*Mustelus canis*) were enclosed in a pressure chamber. The ampulla tubule was cannulated and connected to a pump. Thus, the internal and/or external pressure of the ampulla could be varied over a wide range. Single axons were dis-

sected up to their point of emergence from the ampulla, and electrical activity was led off that point. Generator and action potentials were thus recordable.

At normal atmospheric pressure only a few endings produce generator potentials of sufficient amplitude to fire nerve impulses. But as the pressure of the ampulla is increased, the generator potential is driven progressively to the critical firing level, and impulses are discharged rhythmically and continuously. The main effect of a pressure increment is to increase the rate of rise and the amplitude of the generator potential, and, thereby, to increase the frequency of discharged impulses.

Progressive ablation of ampulla cell structures shows that the generator potential arises in, or close to, the pyramidal cells. The generator structures are excited effectively by either (1) an increase in internal ampulla pressure, that involves stretching; but not by (2) increasing equally and simultaneously the internal and external ampulla pressure, *i.e.*, by a pure pressure stimulus.

The Lorenzinian ampulla seems thus well suited to detect hydrodynamic pressure changes, caused by the fish's own movement or by external objects. In the dynamic phase, its sensitivity is about 11 impulses/second per meter.

Electron microscopy of electrical synapses in the crayfish. A. J. DE LORENZO.

Synaptic junctions in the abdominal ganglia of the crayfish were examined with the electron microscope. Particular attention was directed to axo-axonal junctions which exhibit physiological properties of electrical transmission. The lateral giant to motor junction is characterized by many post-junctional processes which invaginate into the lateral giant or prejunctional fiber. The appositional membranes are separated by a small space approximately 50–75 Å wide. This space is much smaller than that described in other synapses. Each of the appositional membranes is composed of a structural unit consisting of two electron-dense lines, about 25 Å thick, separated by a light zone about 30 Å wide. Occasionally, the unit membranes appear to fuse into a single electron-dense zone about 50 Å thick. Thus, in certain regions, the synaptic membranes exhibit structural modifications at variance with the unit membrane concept. Throughout the region of synapse, however, the synaptic cleft is uniformly 50–75 Å wide.

The axoplasm of the prefiber at the junction is structurally unremarkable, but occasionally rows of mitochrondria are evident. The post-fiber, however, contains large clusters of "synaptic vesicles" which are typically found in the presynaptic terminals of other synapses. Since these synapses have been shown to permit the flow of electrical current only in one direction, it is surprising to find an abundance of synaptic vesicles in the post-synaptic processes. This observation is particularly interesting in view of the current speculation regarding the role of synaptic vesicles in chemical transmission. The role of "synaptic vesicles" in a strictly electrical synapse remains unresolved.

Segmental or septal junctions were also examined. In this junction the synaptic cleft is quite large, often on the order of 400 Å. However, in regions where the pre- and post-fibers interdigitate, the fine processes are separated by clefts about 100 Å wide. It appears that the synaptic cleft in this junction is not uniform in dimensions. Thus, preliminary observations of two electrical junctions suggest differences in ultrastructural organization.

Reversible inhibition of metamorphosis in tadpoles of Amaroecium constellatum by calcium-free sea water. WILLIAM F. LYNCH.

Tadpoles of *Amaroecium constellatum* were taken through four washes of Moore's calcium-free sea water at a pH of 6.2 and were deposited in Stender dishes of the same medium where they became quiescent in a few minutes. All remained in the larval state during seven-hour periods of observation. (The normal maximum natatory period is one hundred minutes.) At the end of seven hours the tadpoles were washed in natural sea water and placed in Stender dishes of this medium. Twenty-four hours later they had formed ascidiozoids with well-developed pharyngeal regions and siphons. The internal organs had assumed the adult axial relationship. Tadpoles that were not removed from the first bath of calcium-free sea water had begun metamorphosis by seven hours, but those in the other baths had not. In the first washing dish metamorphosis had proceeded to the stage in which rotation of the larval axis

had occurred, but the process ceased while the larvae remained in a contracted state. At a pH of 8.1 a similar inhibition of metamorphosis was noted, but this inhibition was no longer reversible when the exposure to calcium-free water lasted for nine hours.

Silicoflagellate populations in the plankton of Cape Cod area, past and present. RAMON MARGALEF.

In the superior layers of the sediment of Cape Cod and Buzzards Bays, skeletons of silicoflagellates are common. The following species of Siphonotestales have been recognized: *Dictyocha fibula* Ehrenb., including var. *pentagona* Schulz, *Distephanus crux* (Ehrenb.) Haeck., *Distephanus speculum* (Ehrenb.) Haeck., including vars. *pentagonus* Lemm., *regularis* Lemm. and *septenarius* (Ehrenb.) Joerg., and *Cannopilus binoculus* (Ehrenb.) Lemm. (probably a simple form of *Distephanus speculum*).

Skeletons increase in abundance from around 16 cm. below the top of all sediment cores towards the surface. Typical *D. fibula* and *D. speculum* make together 99% of the populations. As we approach present time, a shift, similar in both bays, is observed in the composition of populations. *Dictyocha fibula* is almost exclusively represented in the inferior levels. *Distephanus speculum* increases in a regular way from a negligible representation at 8–16 cm. below the surface of sediments up to make 50–60% of the skeletons of silicoflagellates present in the uppermost, soft, layer of the cores.

Dictyocha fibula is a species perhaps of more oceanic character and of rather warm environments; *Distephanus speculum* is more commonly reported from colder water with lower salinity. A differential dissolution of skeletons is unlikely, so that change in the representation of both species may reflect changes in the average composition of plankton accomplished over many years.

The size of the marine diatom Melosira sulcata (Ehrenb.) Kuetz. in the Cape Cod area. RAMON MARGALEF.

In natural populations of several species of *Melosira*, distribution of cells according to diameter shows definite peaks. Such species, all thick-walled, do not experience a noticeable reduction of diameter along successive cellular divisions. Their size distribution suggests the manifestation of a polymorphism.

Different morphs (peaks) seem to have a different selection value under definite environmental conditions and their proportions change in space and in time. Observations in Vigo (NW Spain) suggest that in *Melosira sulcata* low salinity and colder water are, in an independent way, favorable to the high diameter classes.

Forty-two hundred frustules of *Melosira sulcata* have been measured in 5 bottom cores taken in Buzzards Bay and 4000 in two cores obtained in Cape Cod Bay, at depths of 15–29 meters. Diameter ranges from 7 to 50 μ and distribution of cells by diameter offers an image similar to European populations, with peaks at 10, 12–13, 16–17, 20–21, 27, 36–37 μ. Differences exist between bays, between stations and between layers. Shifting of sediments by bottom dwellers does not disturb completely the historical record.

The superior sections of cores show notable parallelism. Considering three levels: a) about 8 cm. below the surface of sediments; b) top of consolidated sediment, ordinarily 3 to 4 cm. below surface; c) unconsolidated materials about 1 cm. below surface, we get the following average diameters: Cape Cod Bay: a) 18 μ, b) 16.7 μ, c) 17 μ; Buzzards Bay: a) 20 μ, b) 17 μ, c) 17.3 μ. The decrease of size, starting at an indeterminate time, is followed by a recent and slight increase. A tentative explanation may be acceptance of an increase of temperature followed by a recent cooling (or decrease of salinity). Salinity rather than temperature may explain the differences between bays.

Pigment composition and productivity as related to succession in experimental populations of phytoplankton. RAMON MARGALEF AND JOHN H. RYTHER.

A type of compound chemostat, made with serially-connected culture vessels, has been employed for the laboratory study of phytoplankton succession. After initial inoculation, fresh

medium is flowed slowly through the system. Soon a steady-state is reached where cell density is independent of the initial state. Then flow can be discontinued and the following measurements made on each vessel: productivity (C^{14} assimilation), cell counts and pigment analyses. The rate of increase, r, in a given vessel is obtained by the expression $r = \ln [1 + (V/C)(C\text{-}B)]$, where C is the cell concentration in the vessel, B the concentration in the previous vessel and V the volumes of the vessel exchanged per unit of time.

In experiments with uni-algal (*Amphidinum*) and mixed (*Syracosphaera, Skeletonema* and *Chaetoceros*) cultures there was consistently observed a decrease in productivity and an increase in the ratio of optical density, 430/665, of acetone extracts of the pigments along the successive elements of the system representing stages of a succession.

Typical results with *Amphidinum* (22° C., 450 foot candles) in a series of three vessels are shown below. In this experiment pH reached 9.5 in some vessels and productivity was based on available bicarbonate only, there being a suspicion that tagged carbonate was not exchanged rapidly with bicarbonate or actually taken up by the algae.

(a) continuous illumination. D430/D665: 3.25, 3.80, 3.85; chlorophyll *a* (μg./10^6 cells): 3.67, 3.65, 2.25; r: 0.88, 0.39, 0.27; productivity (μg. C/10^6 cells/hr.): 1.06, 0.78, 0.37.
(b) 13 hours light/day. D430/D665: 3.55, 3.95, 4.74; chlorophyll *a*: 3.75, 2.70, 1.80; r: 0.78, 0.33, 0.16; productivity: 2.16, 1.86, 1.39.

Note the greater activity of cells subjected to the day-night rhythm.

Purification and properties of Limulus arginine phosphokinase. R. A. MORRISON, A. M. MORGAN AND G. W. DE VILLAFRANCA.

A method for the partial purification of arginine phosphokinase (APK) from the skeletal muscle of *Limulus polyphemus* was investigated. The bulk of the enzyme was precipitated from 50–100% ammonium sulfate saturation of the initial water extract. Dialysis of the residue against 0.001 *M* arginine, pH 8.7 and refractionation with ammonium sulfate between 65–90% saturation resulted in a fraction with a 6.6-fold purification and a 69% recovery. Further fractionation did not increase the specific activity but led only to a decrease in the recovery.

The reaction catalyzed by arginine phosphokinase was followed by assaying for free arginine and was run in the direction:

$$\text{ATP} + \text{arginine} \rightarrow \text{ADP} + \text{phosphoarginine}$$

Activity was found to be directly proportional to time, up to 10 minutes, and also to the protein concentration over a 5-fold range with the substrate concentrations used. The pH optimum is 8.6 and the temperature optimum is in the range of 15–18° C. When the reaction was allowed to proceed at 37° C., the activity was reduced to 50% of the maximum. Although a slight residual activity was found in the absence of added cations, activation occurred in the presence of the following cations in decreasing order: $Mn > Mg >> Fe > Ca > Hg > Co$; Cd inhibited. Optimal activity was noted when the ratio of arginine: ATP: Mg^{+2} was 1:1:1. When the Mg: ATP ratio was greater or less than one, APK activity decreased. The K_m value for arginine at pH 8.7, 25° C. is 2.52×10^{-3} *M*. Substrate specificity was tested with respect to creatine, glycocyamine, taurocyamine, lombricine, and arginine; phosphorylation occurred only with arginine. *Limulus* APK is very labile; it is easily denatured by alcohol, dilution, heat, and by standing at 5° C. Freezing, both in the presence and absence of cysteine, led to a complete loss of activity, while the same treatment in arginine buffer, pH 8.4, showed a 67.7% decrease in activity.

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Comparative aspects of active transport of D-glucose by in vitro preparations of fish intestine. X. J. MUSACCHIA AND S. S. FISHER.

In vitro preparations of everted intestinal sacs were used to measure absorption and transport of D-glucose in segments of intestine from a variety of fish; 33 scup, *Stenotomus versicolor;* 2 sea bass, *Centropristes striatus;* 3 tautog, *Tautoga onitis;* 6 toadfish, *Opsanus tau;* 8 eel, *Anguilla chrysypa;* 71 bullhead catfish, *Ameiurus nebulosus;* 2 perch, *Perca flavescens.*

Active transport of D-glucose was obtained in all the preparations from the fresh-water fishes, in four preparations from eels and in none of the preparations from any of the marine fish.

In a typical experiment, the everted segment of mid-intestine was filled and suspended in teleost Ringer's containing a specific concentration of sugar (5 and 10 mg.%). Preparations were incubated at fish body temperatures (22° to 24° C.) for 20, 30 or 40 minutes. The starting concentrations in the mucosal and serosal fluids were equal. During absorption and transport, sugar was moved against a concentration gradient and increased in concentration in the serosal fluid and concommitantly decreased on the mucosal side. Active transport was uniformly evident in the preparations made from catfish; starting with 10 mg.% glucose, approximately three-fold differences were obtained after 30 minutes. The mean values for 41 preparations changed to 23 mg.% glucose in the serosal fluid and 6.5% in the mucosal fluid. Comparable results were obtained where 5 mg.% glucose was the starting concentration and after 30 minutes, for an average of 30 preparations, glucose in the serosal fluid increased to 13 mg.% and decreased to 2.6 mg.% in the mucosal fluid.

A series of 33 preparations (5 and 10 mg.% glucose at 22° C.) made from scup showed mucosal absorption but no transport of D-glucose. Incubation at elevated temperatures, 37° C., showed very little enhancement of absorption.

Histological preparations showed that the intestinal mucosa retained its integrity during the experimental procedures.

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Some characteristics of active transport of sugars by intestinal segments of Ameiurus nebulosus. X. J. MUSACCHIA.

Absorption and active transport of D-glucose *in vitro* preparations of intestinal segments from the catfish, *Ameiurus nebulosus*, have been reported. In the present study the upper region of the midgut intestine was found to be more efficient in D-glucose transport. Average serosal/mucosal ratios (15 preparations) in 5 mg.% D-glucose were: upper segments, 8.37 ± 1.66; lower segments, 4.93 ± 1.69. At 10 mg.% D-glucose average S/M ratios were: upper segments (19 preparations), 4.74 ± 0.79; lower segments (21 preparations), 2.80 ± 0.40. Routine histological examinations showed shorter and more compact villi in the lower midgut regions, indicating a decrease in total mucosal surface area. Other differences, *viz.* cytological architecture of the mucosal epithelium, distribution of goblet cells, etc., were noted. Such features may have some bearing on the differences in absorption and transport of D-glucose.

A control series of 15 "blank" preparations, wherein the segment was incubated in teleost Ringer's minus the sugar, showed that the amount of glucose endogenously produced ranged from a non-detectable amount in the mucosal fluid to a mean fraction of 2 mg.% in the serosal fluid. These values had no effect in the ultimate determination of glucose.

Low temperatures, 0° to 2° C., inhibit absorption and transport of D-glucose. Preparations were incubated in 10 mg.% D-glucose at low temperatures for 20 minutes and S/M ratios were 1.18 ± 0.04. The same preparations were then incubated at fish body temperature, 22° to 24° C., and active transport was obtained, the S/M ratios being 2.64 ± 0.24. Four preparations were made anoxic with 100% nitrogen for 30 minutes and little or no inhibition of D-glucose transport resulted.

Other sugars, D-xylose, 10 mg.% and 20 mg.%, and D-fructose, 30 mg.% and 50 mg.%, were not transported.

Aided by grant funds from Saint Louis University, Cancer Research Institutional Committee and a Grant-in-aid from the Sigma Xi-RESA Research Fund.

Further studies on the protoplasmic contraction of the marine alga, Chaetomorpha Linum Kützing. W. J. V. OSTERHOUT.

The following experiments form a continuation of the reports published in this Bulletin in 1955 and 1959 and the present *Chaetomorpha* resemble those described in 1959. The purpose is to determine what substances cause the protoplasmic contraction of dead *Chaetomorpha* cells.

A string of living cells was placed in filtered sea water at 50° C. for five minutes. It was then transferred to filtered sea water at 25° C. for one-half hour, after which it was rinsed in distilled water, wiped, and placed in a test solution at 25° C. The cells were at once examined under the microscope and at the start no contraction was detected. But subsequently the protoplasm began to contract. The rate and the degree of contraction in various solutions were compared as follows.

The following solutions are arranged in the decreasing order of their effective action on protoplasmic contraction. HCl, H_3PO_4, both at pH 1.6 > KH_2PO_4, NaH_2PO_4, both at pH 4.5 > phosphate buffer mixture at pH 6 > glass-distilled water at pH 6 > phosphate buffer mixture at pH 8 > borate buffer mixture at pH 8 > Na_2HPO_4, K_2HPO_4, both at pH 9.5 > borax at pH 9.5. Control in filtered sea water showed no contraction, even after 40 hours.

Very rapid and severe contraction occurred in acid solutions at pH 1.6, but much slower and less severe contraction occurred in alkaline solutions at pH 9.5, showing a great difference between the acid and the alkaline solutions. There was less difference between the phosphate and borate buffer solutions at the same pH value. Five-hundredths *M* and 0.01 *M* buffer solutions behaved alike. Sodium and potassium behaved alike.

The contraction was reversed in NaOH solutions at pH 13.

These results indicate that the protoplasmic contraction was brought about primarily by H^+ and secondarily by PO_4^{---} and that the contraction was reversed by OH^-. They may suggest that in a living cell the acids and phosphates formed by metabolism may cause protoplasm to contract, which is reversed by the production of an alkaline substance. This may partly account for the protoplasmic movement.

Experiments with triphosphates will be reported later.

The effects of chloramphenicol on cleavage of Arbacia eggs. EDWARD E. PALINCSAR.

The effects of chloramphenicol on early cleavage of Arbacia were examined to determine the effect of possible inhibition of protein synthesis on the structures and dynamics of cell division. *Arbacia* eggs and sperm were treated with various concentrations (0.2–2.0 mg./ml.) of chloramphenicol in sea water. Cleavage was delayed up to four hours depending upon the concentration, temperature and time of immersion into the chemical. The concentration for blockage of division was 1.5–2.0 mg./ml. Temperatures were experimentally varied from 20–30° C.

Thirty-minute pretreatments of unfertilized eggs and sperm in 0.5–1.0 mg./ml. yielded up to a 34-minute delay in the first cleavage. Fertilization was not affected, but if both treated eggs and sperm were used in fertilization, there was a marked inhibition of cleavage rate and failure to survive past the morula. Five- and 10-minute treatments of the zygotes in 1.0 mg./ml. of chloramphenicol suggested that the period most sensitive to subcritical concentrations is prior to prophase. Blockage in 1–2 mg./ml. is reversible and the inhibition appears to be a freezing of cleavage at the stage when treated. Once the block is reversed, the eggs proceed with the stage at the time of arrest. Using a Warburg respirometer, preliminary data were obtained which indicate no effect of inhibitory concentrations (0.2–0.5 mg./ml.) on oxygen consumption. Subcritical concentrations (0.2–0.5 mg./ml.) did not appear to affect the microscopic appearance, solubility, response to enzymes (trypsin, pepsin, chymotrypsin, ribonuclease) or RNA distribution (azure-B, methyl green-pyronin) of the mitotic apparatus, which was isolated using the dithiodiglycol method. Cytokinesis is unaffected in subcritical concentrations, but there is a marked inhibition of membrane elevation. These preliminary results suggest that the proteins of the mitotic apparatus might be synthesized prior to prophase, since that is where this depressant of protein synthesis has its greatest effect. This possibility is being investigated with isotopes.

The effects of 8-azaguanine and chloramphenicol on the regression-replacement cycle of hydranths. EDWARD E. PALINCSAR AND JOAN S. PALINCSAR.

Substances believed to inhibit protein synthesis and nucleic acid metabolism were used to treat *Obelia* colonies, to determine the phase of the hydranth cycle which might be most affected

and thereby to gain some insight into the cycle transformation. *Obelia* colonies were cultured in aerated sea water (Crowell's method) containing concentrations of 8-azaguanine ranging from 10^{-4} *M* to 10^{-3} *M*, or chloramphenicol concentrations ranging from 0.1 mg./ml. to 1.0 mg./ml. Temperature was constant and observations and counts were made up to 14 days. Concentrations of chloramphenicol above 0.3 mg./ml. and concentrations of 8-azaguanine above 4×10^{-4} *M* were toxic, bringing about regression of the hydranth to the bud stage and eventual disintegration. Eliminating the chemicals resulted in recovery by new upgrowths from the stolon, which thus appeared to be the most resistant. At the lowest concentrations of both chemicals, peculiar, large, elongated, free stolon-like structures grew from the ends of the uprights and occasionally from the sides. These growths were twice the width of a bud, and from 4 to 10 times the length by the second day. The perisarc was not annulated and there was no visible differentiation except a narrowing of the stalk. After four days, small but otherwise normal hydranths differentiated on the ends and sides of the growth. These preliminary results suggest that stages involving differentiation and organization are most sensitive to the chemicals, and that proliferation phases are least inhibited. The possibility of uncoupling growth and differentiation is being further investigated. The chemicals also delayed the regeneration time of cut stems of *Tubularia*. Histochemical studies showed no changes in distribution of RNA and DNA, increased concentrations of succinic dehydrogenase in active hydranths and areas of growth, and increase in calcium concentration during late regression.

The role of moisture and illumination on the expression of the rhythmic behavior of the diatom, Hantzschia amphioxys. JOHN D. PALMER.

The diatom, *H. amphioxys*, surfaces on the sand flats in Barnstable Harbor, Mass., at the time of low tide and migrates downward into the sand when the area is reflooded. In 1951 Fauré-Fremiet reported this rhythmic behavior to persist for six days away from tides in laboratory cultures.

Experiments were conducted during the summer of 1960 to see if any environmental influences on this rhythm could be detected. At high tide sheet metal dams were positioned over previously delimited patches of cells, to retain water in these areas as the tide receded. The resulting cover of water prevented the appearance of algae on the surface, the algae appearing, however, within 30 minutes following draining of the dammed area. The natural flooding tide, or artificial flooding, caused the organisms to migrate downward. In the laboratory, the rhythmic migrations failed to occur under conditions of either constant flooding or drying beyond some critical degree.

Darkness also inhibited the rhythm. Opaque shields placed on the sand before the diatoms normally come up prevent the vertical migration during low tide, and similarly, shields placed at low tide induce the return of the organisms into the sand. That light is the factor involved here is evident from the observation that movements proceed normally under a transparent covering.

Diatoms observed in "preference-chambers" in the field, but isolated from the tidal change, move into the illuminated portion at low tide and into the darkened portion in synchrony with the downward migration of the natural population.

These studies indicate that there is a tidal rhythm in the sign of the phototactic response, it being positive during the time of low tide, and that the expression of the rhythm is influenced by tidal water movements.

Electron microscopic studies of Fucus vesiculosus cytoplasm in summer and winter. JOHNSON PARKER AND DELBERT E. PHILPOTT.

Longitudinal hand sections of *Fucus* midrib 2 inches from the tip were made in January, fixed in buffered OsO_4 at 0° C., dehydrated, embedded in 1 : 3 methyl and butyl methacrylate, and sectioned longitudinally with a diamond knife. Chloroplasts were found clumped together in winter, but lamellae were clearly visible with six layers each (not four as reported in the literature). In cells with slightly shrunken contents, cytoplasm tended to adhere in small strands to the wall, and as a result of this the wall was sometimes slightly expanded, revealing about 25 layers. Good OsO_4 fixation in June was difficult, possibly because of the state of

hydration of the cytoplasm or because of its permeability, a situation which might be related to the lower resistance of *Fucus* to sub-zero temperatures in summer than in winter. $KMnO_4$ at 5% for 10 minutes at 22° C. by Mollenhauer's method gave better results for summer material; chloroplasts were observed resembling those of winter except that they were not clumped. Vacuoles in cytoplasm were more evident in summer and the cytoplasm as a whole was more electron-dense in winter. Cytoplasm clearly passed from cell to cell in medullary elongated cells, as has long been suspected. These connections are relatively massive intrusions of cytoplasm in comparison to the fine strands penetrating the pores of *Macrocystis pyrifera* (brought from the West Coast) sieve plates. Strands in *Macrocystis* appeared to be extensions of endoplasmic reticulum.

Histological investigation of the central nervous system of Clymenella torquata. MABEL C. PATERSON.

In order to understand the significance of regeneration studies in *Clymenella torquata* a more complete knowledge of the histology was considered necessary; consequently an investigation of the central nervous system was undertaken. Anterior regions were serially cut, frontally and sagittally, and stained with a modification of the Holme's buffered silver nitrate technique. The central nervous system is similar to previously described polychaete systems, and consists of a ventral nerve cord which terminates in an enlargement, the subpharyngeal ganglion. This ganglion is united with the paired suprapharyngeal ganglia, located on the dorsal aspect of the pharnyx, by a pair of circumpharyngeal commissures which encircle the pharynx. A number of branches arise from the circumpharyngeal commissures. Each suprapharyngeal ganglion consists of two lobes and receives two major fiber tracts. One of these enters antero-medially and connects with a group of special sensory cells located on the dorsal head plaque, the other enters ventro-laterally and innervates the stomadeal region. The two suprapharyngeal ganglia are connected midway by a bridge of fibers.

The nerve cells within each suprapharyngeal ganglion are peripherally located and appear to be collected in two main groups, anteroventral and dorsal. The fibers of the circumpharyngeal commissure enter the lateral side of each ganglion and therefore separate the two groups of cells. The majority of the cells are of medium size and appear to be bipolar. Their nuclei are round and centrally located. They usually contain one, or occasionally two, eccentrically placed nucleoli. The cytoplasm is granular and some indication of neurofibrils has been observed.

Currently the cellular nature of the subpharyngeal ganglion and the ventral nerve cord is being investigated, as well as possible neurosecretory activity.

Histological investigation of the nephridia of Clymenella torquata. MABEL C. PATERSON AND CARRIE R. KREWSON.

The anatomy and histology of the nephridia of the polychaete *Clymenella torquata* were studied. Four pairs of large nephridia are located laterally in the coelom in segments seven through eleven. Each nephridium occupies portions of two segments, the nephrostome lying in front of the segmental septum and the remainder of the organ immediately posterior. Nephridial openings are observable ventrally at the base of each parapodium.

A plastic reconstruction of a nephridium showed that the organ consists of a funnel-shaped nephrostome opening to the coelom, a long, slightly-coiled, unbranched tubule extending from the nephrostome, a bladder, and a short, straight neck region leading to a nephropore.

The nephrostome is composed of a single layer of ciliated columnar epithelium with a distinct basement membrane. The everted lips of the nephrostome appear in close association with a branch of the sub-intestinal blood vessel. The tubular portion consists of a single layer of irregularly-shaped, ciliated columnar epithelium with a basement membrane; the cytoplasm is densely granulated and highly vacuolated. The bladder and neck regions are formed of a single layer of ciliated squamous epithelium. At the tubule-bladder junction a large tuft of cilia was noted. Ova and sperm packets were observed in the tubule and bladder, suggesting the organ functions in gamete release.

Preliminary histochemical studies indicated alkaline phosphatase localizations in large, sharply-defined granules packed uniformly throughout the cytoplasm in the tubule. No positive alkaline phosphatase reaction was observed in the nephrostome; the bladder showed a positive reaction only at its junction with the tubule. The ciliated inner border of the entire organ is PAS-positive. PAS-positive material was also observed in the basement membrane and in small granules scattered throughout the cytoplasm, particularly in the tubular area. These PAS-positive granules are not comparable to the alkaline phosphatase-positive granules observed in the tubule.

Further electron microscopic observations on the sperm of Limulus polyphemus. DELBERT E. PHILPOTT.

Previous description of the ultrastructure of the sperm of *Limulus polyphemus* reported the head of the sperm as having an acrosome cap and a small "axial" body at the anterior end. An axial core was also seen, traversing the head centrally from the small axial body to the region of the centriole in the distal end of the head. At the time of the first investigation it was not possible to determine if the posterior spiral, consisting of six closely adhering turns, continued up the axial core to the axial body or if the axial core was a process unto itself. Embedding the sperm in epoxy resin and making numerous sections revealed that the posterior spiral does continue up the axial core to the axial body and is thus a continuous structure.

The use of carbon for single molecule visualization. DELBERT E. PHILPOTT.

The method of Cecil Hall for the visualization of individual molecules has been modified in the following way. After the specimen has been sprayed on mica and shadowed with platinum according to his method, carbon is evaporated down onto the specimen from 90° in the place of silicon monoxide. This film is now sufficiently strong to eliminate the next step of flooding the surface with collodion. This saves the time involved in the last step, but more importantly the replacement of silicon monoxide plus collodion as a supporting film increases the brilliance and contrast of the specimen image in the electron microscope. In fact, in some instances the latex particles have been left out of the specimen to be sprayed, both because it was felt advantageous to keep the specimen pure, and because all of the material which is visible on the final print is also visible on the fluorescent screen during specimen scanning. After the carbon evaporation the mica must be placed at once into distilled water in order to float off the prepared specimen. This results in a silver film which easily floats off without the aid of score marks or any other help. However, waiting 24 hours nearly always results in unstrippable films. This stripped film is picked up on 200-mesh copper sheet cut to the size of the floating specimen film, and brought up from underneath, thus mounting the specimen. After blotting the grid material on filter paper, grids are punched out in the usual way for examination. The final result will not be a great deal different from the original and clever method referred to above, but the increased ease and simplicity with which the method can be applied should prove an asset to others, as it has in this laboratory.

Electron microscopic observations of the starfish eyespot. D. E. PHILPOTT AND A. B. CHAET.

A preliminary investigation of the red eyespot located on the short sensory tentacle at the tip of the arm of the starfish, *Asterias forbesi*, was undertaken by means of electron microscopy. The eyespots were dissected from the animal, fixed in osmic acid sea water, embedded in methacrylate and sectioned.

Each of the many eye cups found in the eyespot consists of a cone-shaped layer of red granules, the opening of which is directed towards the outer surface. Electron micrographs indicate two different-sized granules. The smaller, more osmiophilic pigment granules measure about 0.2×0.1 micron in diameter, whereas the larger ones are 1.3×0.8 microns in size. The pigments of the eyespot, B carotene and esterfied astaxanthine, may be represented by the two sizes of granules.

Within the cup two different structures are visible. The one filling the posterior four-fifths of the pigment cup appears quite uniform in nature and is connected at intervals to the lateral walls. The second structure, which is found in the anterior portion, as a layer lying at right angles to the cup's axis, appears to be identical to the outer ectodermal surface of the eyespot itself. This layer as well as the outer surface is about one micron in thickness, and both are covered with micro villi. The layer within the pigment cup may have arisen as part of an invagination of the outer surface, thus explaining the presence of the micro villi which are pointed into the cup. Future attempts will be made to determine if this ectodermal-appearing layer represents a potential lens, and whether the posterior four-fifths of the cup represents neural conducting material.

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Comparative ecology of two species of intertidal amphipods: Talorchestia megalopthama and Orchestia agilis. SARA J. PLATZMAN.

In an intertidal environment humidity is obviously a central factor in determining the distribution of organisms. The intertidal animals would therefore be expected to show adaptations for reduced humidity. Experiments were performed on *Talorchestia megalopthama* and *Orchestia agilis*, two species of intertidal amphipods found near Woods Hole, Massachusetts, at 26.5° C., the approximate summer temperature of this area. At 0% R.H. *Talorchestia* survived for an average of 44 and *Orchestia* 33 minutes. By comparison the members of the wholly marine genus *Amphithoe* survived an average of 13.5 minutes and *Armidillidium vulgare*, a terrestrial isopod, was found by Waloff to live for 429 minutes in 0% R.H. Therefore the R.H. tolerances of *Orchestia* and *Talorchestia* represent a modification toward a land habitat.

The slight difference in humidity tolerance between *Talorchestia* and *Orchestia* is not large enough alone to restrict them to different intertidal habitats. *Orchestia* is found under moist sea-weed at the high water level and is seldom, if ever, present on the surface of the sand. In contrast, the adults of *Talorchestia* live, during the day, beneath 1–5 inches of sand; the lower limit of distribution is 3–4 feet above the water mark of all phases of the tide. Unlike *Orchestia*, they are not confined to the high-water mark. At least part of the populations of *Orchestia* are active day and night. *Talorchestia*, however, is only active on the surface, near the water, at night, although a few juveniles can be found hopping on the surface near the water or in the sea-weed during the day. These observations indicate that behavioral and distributional differences between these two closely related species seem more important than humidity in differentiating their niches.

This work was done as part of the student training program of the Marine Ecology Course.

Analysis of photodynamic effects in lobster neuromuscular preparations. J. P. REUBEN.

Muscle fibers soaked for brief periods in 10^{-4} *M* acridine red, neutral red, or sevron blue responded to illumination with white light by 2 to 10 mV changes in membrane potential. The potentials were of either sign and, when hyperpolarizing, they were reversed by increasing the negative resting potential somewhat more than 15 mV with intracellularly applied hyperpolarizing currents. The membrane resistance, which was increased in the dark after application of the dyes, was lowered during the period of illumination and for some seconds thereafter. The directly evoked responses of the muscle fibers, which were converted from graded responses to spikes by the dye, were reduced during the illumination. Both the excitatory and inhibitory postsynaptic potentials (p.s.p.'s) were also reduced during the irradiation. Return of the amplitudes of the responses to their initial values occurred in the tempo of the return of the membrane resistance.

The frequency and amplitudes of both excitatory and inhibitory miniature p.s.p.'s were, however, greatly enhanced during illumination of the dye-treated preparations. Picrotoxin, which blocked the inhibitory miniature p.s.p.'s, did not block hyperpolarization of the fibers by irradiation. The increased miniature activity appears to be caused by photodynamic effects on the presynaptic nerve fibers. In the muscle fibers, however, the dyes appear to sensitize a

photodynamic conductance change in a component of the membrane that is independent from the electrically excitable or the synaptically activated membrane components. This effect may be analogous to the conversion of previously inert membrane to a chemically excited one in denervated vertebrate muscle fibers. The increased conductance of the photodynamically activated membrane shunts and decreases both the directly evoked responses and the p.s.p.'s.

Electrotonic connections between lobster muscle fibers. J. P. REUBEN.

Depolarizing or hyperpolarizing currents that are applied intracellularly to one (the "proximal") muscle fiber produce changes of the same sign, but of lower amplitude and changed form, in the membrane potential of other ("distal") fibers. The electrophysiological data on the size and form of these electrotonic potentials indicate the occurrence of anatomical structures which form high resistance connections between the two approximately symmetrical resistance-capacity networks of the two muscle fibers. The electrotonic potentials in the distal fiber were attenuated as much as 10-fold in many fibers, but in some there was only 2- to 3-fold attenuation. The greater the attenuation, the more marked was a phase shift between the applied current and the electrotonic potential in the distal fiber. With the larger phase shifts, the potential in the distal fiber continued to grow after the cessation of a brief pulse of current. All-or-none spikes, which were directly evoked in one fiber of procaine-treated preparations, also caused potentials in other fibers, the degree of their attenuation being related to the degree of attenuation of applied pulses. The connections did not exhibit rectification, flow of current apparently being symmetrical in the two directions.

These data and experiments on a model equivalent circuit indicate that the muscle fibers are connected by a link which has a resistance some 3 to 10 times higher than that of the individual fibers. Whether the interconnections are few or numerous between a pair of fibers could not be determined because lobster muscle fibers have a large length constant. There appear to be mutual interconnections in groups of adjacent fibers. However, the electrophysiological evidence of electrotonic connections cannot resolve whether they are formed by syncytial or ephaptic links or by both. In some cases, no electrotonic effects were observed between immediately adjacent fibers, indicating the absence of connections between these pairs. In *Carcinus* and *Cancer* muscles the findings are different.

Actions of cesium ions on the electrically excitable membrane of lobster muscle fibers. J. P. REUBEN AND H. GRUNDFEST.

As is also the case for neuromuscular transmission, short-term exposure of the fibers to Cs^+, with or without removal of K^+, did not affect the electrically excitable membrane. Soaking the preparation for 10 hours or more in K^+-free Ringer's solutions containing 15 to 50 meq./l. of Cs^+ did not markedly change the membrane potential or resistance from their values in K^+-free solutions without Cs^+. Direct stimulation still produced small graded responses, although the large excitatory postsynaptic potential of the Cs^+-soaked preparations caused a large spike-like response. Profound changes in the ionic conductance mechanisms of the electrically excitable membrane could nevertheless be shown on additional treatment of the preparations with various agents. Thus, while increasing external K^+ caused depolarization, the change was only about 30 mV/decade change in K^+. On changing the medium from zero K^+ to 30 meq./l., the resistance fell only 3- to 4-fold, while in untreated muscle fibers the change is about 100-fold. Unlike untreated preparations, those soaked in Cs^+ tolerated exposure to a Ca^{++}-free medium for long periods. On diminishing the external Ca^{++}, the directly evoked responses increased in size. However, there was also a large, reversible decrease in membrane resistance, though only a small decrease in resting potential.

The addition of procaine (10^{-3} to 10^{-2} w/v) to normal preparations converts their graded responses to brief, all-or-none spikes. In Cs^+-soaked preparations, application of the drug caused indefinitely prolonged spikes, that were followed in some experiments for more than 30 minutes and terminated only by applying strong hyperpolarizing currents. The conductance during the spike was up to 20 times higher than that of the resting membrane.

The overt effects of Cs^+ in general resemble and are synergistic with those of Ca^{++}, but

they are more extreme under some experimental conditions and less so under others. These similarities and differences indicate that Cs^+, like the alkali earth ions, has several modes of action. They also indicate that the several molecular mechanisms which are presumed to underly these effects are independent to some degree.

Further analysis of the conversion of graded to all-or-none responsiveness in the electrically excitable membrane of lobster muscle fibers. J. P. REUBEN AND H. GRUNDFEST.

The normal graded activity of lobster muscle fibers can be converted to all-or-none by various agents. These effects can be analyzed in terms of the various changes of ionic conductances and electrode properties of the electrically excitable membrane (see Grundfest, this issue).

The conversion by cocaine (10^{-4} to 10^{-5} w/v) and procaine (10^{-3} to 10^{-2} w/v) occurs with only small changes in resting membrane potential and resistance. The spikes are brief compared with those produced by alkali earth or onium ions. The drugs therefore probably only decrease the outward current during the response, by inactivation of K^+-conductance. Cocaine in higher concentrations abolishes activity, probably by also blocking inward current. In contrast, the conversion of the membrane to all-or-none responsiveness by d-tubocurarine is associated with hyperpolarization and increased resting membrane resistance. Thus, this drug also exerts another of the actions of the divalent alkali earth ions, block of resting K^+-conductance. However, enhanced Na^+-conductance does not develop, and the spikes are brief. Methylene blue, sevron blue, acridine red and neutral red appear to act in the same way as does d-tubocurarine. Procaine, in combination with other agents (Ba^{++}, Cs^+, phenethylamine derivatives, or serotonin-like compounds), causes spikes that may last for 30 minutes or more. Thus, the combined action of the agents also blocks Na^+-inactivation to an extreme degree.

Procaine and d-tubocurarine convert all-or-none responses of other electrically excitable membranes to graded activity. Thus, in their actions on different cells, various agents may have "excitant" or "depressant" effects, depending upon the conditions that prevail in the specific membrane. The nature of the effects probably derives from the degree and the critical ratios to which the different factors of electrical excitability participate in each response, and from the degree in which each component is affected by the various agents. The latter variable is probably determined by different details of molecular structure that are as yet unknown.

Inhibitory and excitatory miniature postsynaptic potentials in lobster muscle fibers. J. P. REUBEN AND H. GRUNDFEST.

Miniature potentials of synaptic origin occur spontaneously in lobster muscle fibers, due to activation of the inhibitory as well as the excitatory synaptic membranes. The inhibitory miniatures may be of either sign, depending on electrochemical conditions. Both activities are probably due to summation of transmitter actions of many presynaptic terminals. Inhibitory miniatures of up to 1 mV have been observed and the excitatory tend to be still larger. The inhibitory activity is abolished by picrotoxin and the amplitudes of the excitatory miniature potentials are then greatly augmented. The frequency and amplitudes of the miniature potentials are modified by applying various agents to the preparation. NH_4Cl (15 meq./l. or more) at first causes an increase in the frequency of both types of miniature potentials. Later, both are few in number, but large and prolonged. When the inhibitory are eliminated by picrotoxin, the excitatory potentials grow to very large amplitudes. Large, spontaneous potentials, principally or exclusively of the excitatory variety, are induced after serotonin. The potentials occur independently in different fibers of the same muscle, though all are innervated by the same axon. Thus, the independent origins of the spontaneous potentials indicate that they probably occurred independently of an axonal spike, and that the transmitter actions occurred independently in different presynaptic terminals.

Presence or absence of miniature potentials provides a criterion for the mode of action of various agents on the different components of the neuromuscular system. Thus, a very powerful blockader of lobster neuromuscular transmission, the purified dinoflagellate "clam

poison," does not eliminate either variety of miniature potentials, nor the larger potentials induced by NH_4Cl. Therefore, the poison must block arrival of the spike at the presynaptic terminals, while activity of the latter is unimpaired.

The action of cesium ions on neuromuscular transmission in lobster. J. P. REUBEN AND H. GRUNDFEST.

Addition of Cs^+ (15 to 50 meq./l.), with or without removal of K^+ in *Homarus* Ringer's solution, had no apparent effect on short-term exposure of the preparation to this ion. Profound alterations were produced, however, on soaking the preparations for 10 to 20 hours in K^+-free solutions containing Cs^+. The excitatory and inhibitory postsynaptic potentials (p.s.p.'s) of the muscle fibers were greatly augmented when recorded in a K^+-free medium, with or without Cs^+. A single stimulus to the excitatory axon evoked a large, spike-like potential and a vigorous twitch. The i.p.s.p. evoked by a single stimulus to the inhibitory axon was maximal, or nearly so. The degree of facilitation of the i.p.s.p.'s was correspondingly small.

The sensitivity of Cs^+-treated fibers to GABA was about like that of untreated preparations. Thus, the inhibitory synaptic membrane had not become more sensitive to this drug. The reversal potential was unchanged and the augmented i.p.s.p.'s could not have resulted from changed electrode properties of the synaptic membrane. The reversal potential of the e.p.s.p.'s was between 20 and 30 mV, inside negative, and probably had not been changed by the ion, but a direct test for the chemical sensitivity of the excitatory synaptic membrane was not available. It is unlikely, however, that the augmentation of both p.s.p.'s by Cs^+ was due to actions on the presynaptic terminals, increasing the transmitter action of both.

When K^+ was present in the medium, the p.s.p.'s were markedly reduced in size. The decrease of the i.p.s.p. was directly related to the decreased membrane resistance and the changed membrane potential of the muscle fibers. The reduction in the e.p.s.p.'s was much greater. When the external K^+ was double that of *Homarus* Ringer's solution the membrane resistance had decreased 3- to 4-fold, but the e.p.s.p.'s were diminished about 10-fold. The effect of K^+ on the e.p.s.p. therefore indicates that K^+ blocks competitively the Na^+ channels opened during activity of the synaptic membrane.

Properties of indefinitely prolonged spikes of lobster muscle fibers. J. P. REUBEN, R. WERMAN AND H. GRUNDFEST.

When treated with various agents (Reuben and Grundfest, this issue), lobster muscle fibers produce directly evoked responses that are spikes with amplitudes up to 120 mV and durations of more than 30 minutes. The membrane conductance during the response was increased 3- to 20-fold. Responses with conductances in the lower part of this range could be terminated by applying strong inward currents. The termination was associated with an increase in membrane resistance, and the change in potential had a time course resembling that of hyperpolarizing responses. In spikes with high membrane conductance, the potential returned to its plateau value even after strong inward currents had hyperpolarized the membrane for several seconds. This return may have been a manifestation of anodal break excitation.

Spontaneous termination of the spike was frequently associated with up to 15 mV hyperpolarization, lasting 15 seconds or more. In other cases it occurred through stages of intermediate plateaus, each lasting several seconds. Low frequency oscillations between metastable states often occurred and sometimes lasted more than 10 minutes. The durations of the individual oscillations varied. At different times during a single sequence their forms resembled those of vertebrate cardiac potentials, "upside-down" potentials, or hyperpolarizing responses. They ranged in amplitude from less than 1 mV to about 50 mV.

These different effects are ascribable to the operation of different combinations of the various ionic conductances and the consequent electrode properties of the electrically excitable membrane (Grundfest, this issue). Local changes in these relations, which probably reflect non-uniformities in different regions of the membrane, add further complications. Their various manifestations in lobster muscle fibers suggest a possible mechanism for "pacemaker" activity in other types of cells.

Further studies on the biochemical effects of x-radiation on Tetrahymena pyriformis. JAY S. ROTH, ELEANOR HENRY AND CAROL WIERCINSKI.

Three-day cultures of *Tetrahymena pyriformis* W, grown in proteose-peptone plus yeast extract, were collected by gentle centrifugation and thoroughly washed. Cells were suspended in water or buffer and irradiated with from 300,000–600,000 r. In some cases cells were irradiated in the presence of metabolites, or metabolites were added immediately after irradiation.

Growth studies using an inoculum of approximately 2×10^5 cells indicated that no cells survived 600,000 r. With lower doses there was a lag period in the growth of irradiated cells, the length of the lag being roughly proportional to the dose, but thereafter irradiated cells grew at the same rate as controls. When cells were irradiated with up to 600,000 r in 0.05 *M* phosphate buffer, pH 7.4, there was either a slight stimulation of respiration or no change. However, irradiation in water resulted in the depression of respiration from 50–90% at 600,000 r. Addition of thioctic acid (0.5 mg./ml.) to cells during or immediately after irradiation with 300,000 r had no significant effect on respiration, nor did it appreciably affect the increase in respiration observed when irradiated cells were treated with 0.05 *M* pyruvate. Lactate (0.05–0.2 *M*) stimulated the respiration of control cells, and the oxidation of lactate by irradiated cells was increased over that of controls by from 20–100% as long as two hours post-irradiation with 300,000 r. The amount of increase was dependent on the time post-irradiation and the concentration of lactate. The oxidation of lactate was depressed, nevertheless, in a cell-free homogenate of *Tetrahymena* irradiated with 300,000 r, as well as in a homogenate prepared from irradiated cells.

The effects observed appear to be explained best on the basis of an increase in permeability of the cell membrane, and possibly mitochondrial membranes to lactate and pyruvate after irradiation. (Supported by a grant from the U. S. Atomic Energy Commission.)

X-ray-induced mitotic delay in the Arbacia egg. RONALD C. RUSTAD.

Since multipolar divisions appear to define damage to a system controlling the multiplication of asters, further studies on the parallelism between the induction of multipolar spindles and mitotic delay have been conducted. A pre-fertilization recovery period reduces both effects in irradiated eggs. The same mitotic stage (early streak) is maximally sensitive to the induction of both effects. Direct tri- or quadripolar second divisions indicate that the effect is general and not a special property of fusion nuclei.

The preparation for a multipolar division includes a previously undescribed mitotic stage: a double streak.

The experimental induction of polyspermy has been used to analyze the additivity of x-ray-induced mitotic delay. The presence of several irradiated sperm neither lengthens nor shortens the period of mitotic delay. When unfertilized eggs are irradiated and then made polyspermic, some of the eggs divide sooner than monospermic ones.

The division cycle of *Arbacia* eggs becomes insensitive to the induction of mitotic delay by either acridine orange or irradiation at approximately the same time. Treatment of sperm with either acridine or x-rays extends the x-ray-sensitive portion of the mitotic cycle, which corresponds cytologically to early streak, the period of multiplication and separation of the centrioles.

Low dose x-irradiation and the possibility of accelerated root growth. B. P. SONNENBLICK AND JOHN KEOSIAN.

It would be of theoretical and practical significance to determine definitively the existence of, and then induce as desired, the so-called "stimulating" effect of radiation on intact living organisms. An influence has been claimed by some earlier investigators and sporadically by recent ones, but others deny its existence. Would exposure of bulbs to doses circa 100 r influence subsequent rooth growth? *Allium* bulbs of heterogeneous origin (heterogeneity was desired) were exposed in exploratory tests to x-rays of 85 and 182 kvp, dose rates of 45 r and 350 r/minute. Germination began 12 hours later and test temperatures recorded from

24.1–26.3° C. Periodic observations were made and after 14 days all roots removed and their lengths measured. Almost 11,000 roots from approximately 400 irradiated and unexposed bulbs have been measured.

The results are equivocal. It is possible to record with doses of 50–100 r an accelerative influence, or the absence of such, according to the criterion used. Criteria may be (a) total number of roots, (b) average root length, (c) percentage of roots surpassing an arbitrarily chosen length, (d) percentage of bulbs germinating. In a given experiment an effect may be indicated by one or more of the criteria but not by others. Employing criteria (b) and (c) above, it would appear that low dose irradiation could have stimulated root growth relative to controls in two of six tests. It will be interesting to note future results if some variables in our complex system, or with other projected biological material, are eliminated.

Duration of the experiment is an important consideration. Roots from exposed bulbs may, during the first week, manifest an accelerated growth rate which might be regarded as stimulation were the test ended at that time. Control roots can often bypass the others in length during the second week. Early acceleration may thus be a spurious phenomenon.

Comparative effects of x-ray and ultraviolet radiation of gametes on the developing sea urchin Arbacia. CARL CASKEY SPEIDEL AND RALPH HOLT CHENEY.

Effects of x-ray and 2537 Å ultraviolet (UV) radiation of Arbacia gametes were analyzed by observing the subsequent stages of development after mixing various combinations of radiated gametes. In addition to fertilization of normal eggs with normal sperm, 8 gamete combinations were studied: (1) x-rayed eggs with normal sperm, (2) UV-rayed eggs with normal sperm, (3) normal eggs with x-rayed sperm, (4) normal eggs with UV-rayed sperm, (5) x-rayed eggs with x-rayed sperm, (6) UV-rayed eggs with UV-rayed sperm, (7) x-rayed eggs with UV-rayed sperm, (8) UV-rayed eggs with x-rayed sperm. Radiation doses ranged from mild to severe. Fertilization occurred with all gamete combinations. Development was watched from the moment of insemination to the larval pluteus stage. Time-lapse cinephotomicrography recorded early viscosity changes and the sequence of protoplasmic movements culminating in irregular cleavages.

Certain differences in the progeny resulting from the 8 combinations of radiated gametes were ascribed to differences in penetration of the two kinds of radiation, deep with x-rays and shallow with UV-rays. Graded quantitative results, based upon both amount of developmental retardation and degree of injury, were more uniform after graded x-ray dosages than after graded UV exposures. Greater variation in UV-rayed egg experiments resulted from the different amounts of radiation that reached the near (to UV source) and far sides of the exposed eggs, combined with the variable position of the nucleus in individual eggs. Differences in radiation penetration also affected comparative radiosensitivity. Thus a short exposure of sperm (small-sized cells) to UV-rays caused developmental retardation and injury equal to a longer exposure of eggs (large-sized cells), 4–16 times as long. In contrast an x-ray dose to sperm was equal to a dose of only 1.5–2 times as much to eggs.

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Developmental potential of the chick blastula (unincubated blastoderm). NELSON SPRATT AND HERMANN HAAS.

Typical development (through 10± somite stages) occurs in almost all unincubated blastoderms explanted upper surface against a new yolk-albumen extract agar medium. This method has made possible for the first time an extensive analysis of the blastula stage (24–28 hours post-fertilization) in the freshly-laid chick egg.

The bilaterally symmetrical organization of the unincubated blastoderm, although quite evident in a cell density gradient and pattern of lower layer morphogenetic movements, is in no sense irrevocably determined either in its position or direction. Complete transection and longitudinal section, partial longitudinal section with a sizable connecting bridge of pellucid and opaque area cells, separation of the prospective posterior quadrant from the remaining three-fourths of the blastoderm, etc., in short, any cut through or into the system stimulates

regenerative growth which tends to transform the outline of a fragment into a circle, *e.g.*, like that of the intact whole blastoderm. This in turn may bring about either: (1) a change in direction of the median plane of lower layer movement (and consequent position of the embryonic axis upon the blastoderm), (2) complete reversal in polarity of the movement pattern (and of the direction of the embryonic axis), (3) induction of one or two new secondary bilateral patterns of movements (one or two complete secondary embryos) or (4) division of the normal axial pattern into two bilaterally symmetrical movement patterns (two complete embryos).

The unincubated blastoderm is thus capable of great regulation and regeneration and consequently exhibits the properties of an embryonic field. Any part of the system large enough (exceeding about ¼ of the original mass) is able to form a complete, bilaterally symmetrical embryo morphologically and functionally equivalent to a normal 10± somite stage. About 80 twins and 7 triplets have been obtained to date.

Whole-mount preparations of fish lens epithelium from various species. B. Dobli Srinivasan.

Whole-mount preparations of epithelium from the crystalline lens of rabbit, rat and frog have proven useful in studies on cellular proliferation in normal and injured tissues (Harding *et al.*, 1960). The unique characteristics of the lens (*e.g.*, its avascularity, lack of a nerve supply, enclosure within a membrane, presence of epithelium as a single layer of cells) have been shown to be of advantage in studies on the relationship between injury, and DNA synthesis and mitosis in the rabbit lens (Harding *et al.*, 1959). It would be of interest, for a number of reasons, to extend this study to include the lenses of cold-blooded animals, and the present report indicates that whole-mounts of the entire layer of lens epithelium can be prepared from the following species: sea bass (*Centropristes striatus*), dogfish (*Mustelus canis*), dusky shark (*Carcharhinus obscurus*), goose fish (*Lophius americanus*), sea robin (*Prionotus carolinus*), tautog (*Tautoga onitis*), scup (*Stenotomus versicolor*), and skate (*Raja erinacea*).

A procedure previously described (Harding, 1960) was followed in making the whole-mounts. In all cases essentially complete layers of epithelium could be prepared. They consisted of a single layer of cells. Stainability with Harris' hematoxylin varied from species to species. Good results were obtained with dogfish, dusky shark, goose fish and skate. Mitotic figures were found in preparations from dogfish, sea bass, scup, tautog and skate. Mitotic figures, in these species, were found scattered over a relatively wide area as compared with the peripheral band seen in the rabbit and rat previously described. Lenses from goose fish and dusky shark were not fixed immediately after death of the animals and mitotic figures were not seen in these specimens.

It is concluded that the entire layer of lens epithelium, in the form of a whole-mount, can be prepared from a number of species of fish, and that a few of these species, namely dogfish and skate, appear to be very suitable for studies on cellular proliferation.

Extraction of chlorophyll from marine plankton algae with acetone and methanol. Carol Sterns.

Standard spectrophotometric methods for the quantitative determination of chlorophyll are based upon the specific absorption of pure acetone solutions of the pigment. This technique has been widely adapted in ecological research for the measurement of chlorophyll in natural plankton populations. There are many indications, however, that chlorophyll extraction of marine plankton algae with cold acetone as a solvent is far from complete. This is particularly true of certain species (*i.e.*, many of the green algae) with tough, resistant cell walls. Higher chlorophyll values are consistently obtained if the cells (millipore-filtered or centrifuged from suspension) are subjected to mechanical grinding or sonification. Neither of these latter techniques is practical for field analyses or for measurement of large numbers of samples.

On the other hand, hot methanol, long used for chlorophyll extraction of higher plants, appears to be considerably more effective than acetone for pigment extraction of marine phytoplankton. While some modification of the standard formulae for relating optical den-

sities at various wave-lengths to concentrations of the different pigments may be necessary, it is noteworthy that the red absorption peaks of methanol and acetone solutions of chlorophyll (664 vs. 661 mμ) are nearly the same, and that relative absorption at the respective peaks is only about 3% higher for methanol than for acetone.

A comparison of the optical densities at 665 mμ of cold 90% acetone and hot methanol extracts (methanol hot initially, extraction for 24 hours in dark and refrigeration) of a variety of organisms gave the following results (shown as O.D. methanol/acetone): *Nannochloris atomus* (green) 1.96; *Synechococcus* sp. (bluegreen) 1.60; unidentified diatom 1.18; *Dunaliella euchlora* (green) 1.00; *Amphidinium carteri* (dinoflagellate) 1.00; *Pyramimonas* sp. (green) 0.94; natural plankton from Vineyard Sound, 1.29. This work was carried out as a part of the student training program of the Marine Ecology Course.

Further inquiry into the mechanisms of aqueous humor formation in dogfish. WILLIAM STONE, JR., DANA FELDSHUH, JUDITH LEINING AND R. F. DOOLITTLE.

The osmotic pressure of the aqueous humor of the smooth dogfish (*Mustelus canis*) is lower than the osmotic pressure of its blood plasma. The osmotic pressures of both plasma (960 milliosmoles) and aqueous humor (935) are higher than that of the environmental sea water. It has been suggested that water moves across the cornea from the sea, thereby diluting the aqueous humor with respect to the plasma from which it is derived. If this were so, differences in the osmotic pressure of the sea water bathing the cornea should be reflected in the degree of dilution of aqueous humor. Smooth dogfish were subjected to sea waters of varying tonicities for times ranging from one to six hours. When the osmolarity was lowered as much as 100 milliosmoles below normal, neither aqueous humor nor plasma exhibited any significant change. When the osmolarity was raised, the plasma and aqueous humor osmolarities increased in parallel, the difference between them remaining constant. Furthermore, when the formation of aqueous humor was arrested by intravenous administration of Diamox (acetazoleamide), the aqueous humor osmotic pressure increased, in the limit reaching that of the plasma. These phenomena were interpreted as meaning that the difference in the osmotic pressures of dogfish aqueous humor and its plasma is independent of any water entering the eye across the cornea. A preliminary investigation of the enzymatic profile of the dogfish ciliary body confirmed a high level of carbonic anhydrase, inhibition of which could be effected by both *in vivo* and *in vitro* Diamox administration. The Q_{O_2} of dogfish ciliary body is higher than that of its retina, when compared on a dry weight basis. In addition, the two tissues respond differently to the presence of substrate (glucose) during respiration studies, the ciliary body Q_{O_2} being higher with glucose added (3.08) than the ciliary body endogenous (1.86), whereas retina endogenous is higher (1.13) than when added glucose is present (0.95).

Separation and isolation of Spisula embryo cells. CORNELIUS F. STRITTMATTER AND PHILIPP STRITTMATTER.

A technique has been developed for separation and isolation in mass of individual cell types from embryos of *Spisula solidissima*, in order to permit studies on enzyme components of various cell types during early embryonic development. The technique involves dissociation of embryo cells in calcium-free medium and subsequent isolation of cell types of different sizes by density gradient centrifugation.

Spisula embryos are harvested at a selected stage of development, washed twice with cold 0.52 *M* NaCl–0.02 *M* Tris buffer, pH 8, (NaCl) to remove calcium, then suspended in cold 0.07 *M* sucrose–0.02 *M* Tris buffer, pH 8 (SM). On prolonged standing in this medium, the cells of the embryo dissociate slowly, but complete dissociation is rapidly achieved by adding *ca.* 0.0005 *M* Versene and and gently agitating the suspension. The separated cells retain a normal appearance and are capable of further cleavage.

The subsequent density gradient centrifugation may be illustrated by the isolation from 4-cell *Spisula* embryos of the large D cell and a mixture of the small A, B and C cells. The suspension of dissociated cells in sucrose medium is diluted with NaCl medium to give a

60 SM : 40 NaCl mixture. A 2-ml. aliquot of this mixture is layered over 10 ml. of 75 SM : 25 NaCl in a 15-ml. tube and centrifuged at *ca.* 70 *g* for three minutes. A 2-ml. fraction drawn off from the gradient boundary contains an 80–90% pure mixture of A, B and C cells in *ca.* 20% yield, while a 2-ml. fraction from near the bottom of the tube contains 90% D cells in *ca.* 20% yield. The purity of each fraction may be increased by a second centrifugation.

The density gradient centrifugation is applicable to isolation of cell types from dissociated embryos of other developmental stages by appropriate modifications of medium densities and of the duration and force of centrifugation.

Electron transport in eggs, developing embryos and adult tissues of Spisula solidissima. CORNELIUS F. STRITTMATTER, PHILIPP STRITTMATTER AND CAROLYN BURDICK.

Enzymes of electron transport in unfertilized eggs and in adult heart and liver of *Spisula solidissima* have been studied by spectrophotometric assay of homogenates and fractions obtained by differential centrifugation. Unfertilized eggs showed a high activity of DPNH-cytochrome *c* reductase (10-12 μmoles electrons/min./ml. eggs), DPNH-ferricyanide reduction and cytochrome oxidase (2–4 μmoles electrons/min./ml. eggs), appreciable activities of succinic dehydrogenase (0.13–.20 μmoles electrons/min./ml. eggs) and TPNH-cytochrome *c* reductase, and a lower DPNH oxidase activity. Adult tissues showed somewhat similar but distinctive patterns. Unfertilized eggs, heart and liver each contained a particulate fraction resembling mammalian mitochondria in (1) centrifugal behavior, (2) absorption spectra, which indicated the presence of cytochromes *a* (and a_3), *b* and *c*, (3) oxidative activities, including essentially the total cell content of cytochrome oxidase and antimycin A-sensitive succinic dehydrogenase and a large portion of the DPNH-cytochrome *c* reductase activity, and (4) staining reactions, being the major site of cyanide-sensitive Nadi and Janus green reactions.

Tests on homogenates from fertilized eggs and embryonic stages up to 22-hour swimming forms showed no marked changes from unfertilized eggs in cytochrome oxidase, DPNH-cytochrome *c* reductase and DPNH-ferricyanide reduction. As these activities were in excess of values reported in the literature for oxygen consumption of *Spisula* eggs and early embryos, it appears that these terminal electron transport capacities are not limiting factors involved in the reported increase of oxygen consumption rate during early stages of *Spisula* development.

The ratio of succinic dehydrogenase to cytochrome oxidase activity was markedly higher in adult heart than in unfertilized eggs or early embryos. This change may possibly reflect a differentiation of mitochondria.

Cardiovascular and respiratory activity in dog fish. FREDERICK N. SUDAK AND CHARLES G. WILBER.

Squalus acanthias and *Mustelus canis* were pithed anteriorly to the mesencephalic region and posteriorly from the 14th to 18th vertebrae. Blood pressures were measured with pressure transducers. Respiratory activity was recorded directly from the branchial muscles. Electrocardiograms were recorded using bipolar electrodes. Deep body temperatures were maintained at 23.0° C. ± 0.5° C. Average heart rates at this temperature were 46.8 beats/minute in *Mustelus*, 28.5 beats/minute in *Squalus*. The heart rate Q_{10} (23° C. to 33° C.) in *Mustelus* was 3. EKG's in *Mustelus* showed a P-R interval of 0.25 sec., QRS of 0.07 sec. and QT interval of 0.58 sec. The ST segment was isoelectric and the T wave biphasic. Cardiac rhythmicity bore a phasic relationship to respiratory activity in both species. RR intervals were constant when the ratio between respiration rate and heart rate was 1 in *Mustelus* and 2 in *Squalus*. Arrhythmias of various degrees were observed whenever the two cycles fell out of phase. Respiratory activity occurring during the middle third of a given cardiac cycle consistently prolonged the diastolic period of that cycle. Respiration rate in *Mustelus* was 48.7/minute, in *Squalus* 69.0/minute.

The average mean perfusion pressure from the ventral aorta to the venous side of the circulation was 22.4 mm. Hg. End diastolic intraventricular pressure varied from minus 0.7 to 0 mm. Hg with the pericardium intact. Average blood pressures in the ventral aorta were:

systolic 29.5 mm. Hg, diastolic 21.2 mm. Hg and pulse pressure 8.2 mm. Hg. The mean blood pressure showed periodic variations, resembling Meyer waves described in mammals. The periodicity was 2.8 cycles/minute in *Mustelus* and 0.8 cycles/minute in *Squalus*. The magnitude of the pressure change was 1.4 mm. Hg.

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Cardiovascular responses to hemorrhage in the dog fish. FREDERICK N. SUDAK AND CHARLES G. WILBER.

Cardiovascular and respiratory responses to acute hemorrhage were studied in *Mustelus canis* and *Squalus acanthias*. All animals were pithed anterior to the mesencephalon and posteriorly from the 14th to 16th vertebrae. Blood pressures were recorded directly from the ventral aorta. Electrograms obtained from the branchial muscles were used to record respiratory activity. Blood pressures and respiration were recorded before, during and after the removal of blood from the caudal vein, and following reinfusions of blood into the ventral aorta. Systolic and diastolic pressures decreased linearly with the increase in blood deficit. Systolic blood pressure decreased an average of 1.8 mm. Hg/ml. of blood lost, while the diastolic pressure decreased an average of 1.0 mm. Hg/ml. of blood lost. A similar pressure-volume relationship was obtained when blood was reinfused. Mean blood pressures were reduced approximately 50% after removal of 5–7 ml. of blood (10% of estimated total blood volume) from a 700-gram fish. Changes in heart rate and respiration rate were inconsistent. In general, the cardiovascular system in both species behaved like a simple pressure-volume system in response to blood loss. No evidence was found to indicate the presence of a compensatory mechanism against decreases in blood pressure resulting from reduction of blood volume.

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Uptake and release of calcium-45 by Fucus vesiculosus. ELIJAH SWIFT AND W. ROWLAND TAYLOR.

Five to seven grams (dry weight) of whole young thalli of *Fucus vesiculosus*, without macroscopic epiphytes, were incubated in sea water in 260-ml. bottles maintained at 22° C. and under constant illumination (700 to 1300 foot candles, depending on the experiment). The bottles contained about 1 μC. of $Ca^{45}Cl_2$. The uptake or release of isotope was followed by removing aliquots of sea water, plating in the usual manner, and assaying in a gas flow counter.

The algae appeared to take up radiocalcium rapidly, most of the uptake occurring in the first hour. The isotope did not appear to be concentrated since calculations from Vinagradov's tables indicate the amount of isotope taken up was of the order of magnitude that would be expected if the radiocalcium in the sea water came into equilibrium with the calcium in the algae.

Fucus vesiculosus maintained in sea water containing calcium-45 was rinsed, and placed in millipore-filtered sea water to which no isotope had been added. In 24-hour experiments, 90% of the radiocalcium appearing in the medium was released in the first two hours. Similar studies were done with thalli that had been used in uptake experiments. If it is assumed that the radiocalcium in the thalli came into equilibrium with the medium in both the uptake and release experiments, the calculated and observed values check within 10%. These techniques did not show a significant difference between *Fucus* maintained in the dark or illuminated, in either the uptake or release experiments. These data indicate there is a rapid turnover of calcium by *Fucus vesiculosus*. (Partly supported by contracts between the Chesapeake Bay Institute, The Johns Hopkins University and the A.E.C. and O.N.R.)

The effect of photosynthesis by benthic macroalgae on the titration alkalinity of sea water. W. ROWLAND TAYLOR.

Six species of benthic macroalgae have been examined to determine the effect of their photosynthetic and respiratory activity on the alkalinity and pH of the sea water medium. The algae, 0.5 to 1 gram wet weight depending on species, were incubated in series of 125-ml.

light and dark bottles at constant temperature (21° C.) and illumination (1800 foot candles). Changes in the titration alkalinity and pH were noted and rates of photosynthesis and respiration were followed by oxygen measurements.

When *Ulva lactuca* or *Enteromorpha intestinalis* was incubated in this manner, the pH of the medium increased from 8.0 to 9.0 in about six hours but the alkalinity did not change. On continued incubation, the pH rose to 10.0 and the titration alkalinity decreased from initial values of 2.20 to 1.50 milliequivalents/liter. Oxygen determinations indicated continued photosynthesis at these high pH levels. Photosynthesis by *Ceramium rubrum, Chondrus crispus, Chorda filum* and *Fucus vesiculosus* resulted in an increase in pH to maximum values of 9.0–9.2. With one exception, there was no change in the alkalinity. In the case of the *Fucus* there was a small decrease in the alkalinity during the latter part of extended runs. Respiration by all of these algae resulted in a decrease in pH but no change in alkalinity. Preliminary experiments, using sea water in which the partial pressure of CO_2 had been lowered, indicated the rate of oxygen production by the *Fucus* and *Enteromorpha* species may be dependent on the concentration of dissolved CO_2.

These findings suggest that these algae probably use dissolved CO_2 as their carbon source under their usual environmental conditions. However, the two Chlorophyta can use bicarbonate (or carbonate) when growing at high pH. (Partially supported by contracts between The Johns Hopkins University and the A.E.C. and O.N.R.)

Uptake of iron-59 by marine benthic algae. W. Roland Taylor and Eugene P. Odum.

Exploratory experiments have been undertaken to investigate the uptake of iron-59 by marine benthic algae. A typical experiment consisted of placing a weighed amount of an alga in each of a series of stoppered bottles or flasks filled with sea water. Suitable amounts of $Fe^{59}Cl_3$ were added to the containers; they were maintained at constant temperature and either illuminated or darkened as required. The uptake of the isotope was followed by removing aliquots of the water and counting with a scintillation detector. In some experiments, pH and oxygen production were also measured. Such studies were complicated by adsorption of the isotope on the walls of the container, and it was necessary to apply corrections obtained from blanks or add chelating agents to the medium.

The isotope was taken up by *Ceramium rubrum* and *Enteromorpha intestinalis* rapidly and there was little loss of the isotope when algae were removed from the radioactive medium and placed in fresh sea water. A rough estimate of the concentration factor would be about 100 to 300. Addition of 30 mg./liter of the chelating agent ethylenediaminetetraacetic acid was found to prevent the loss to the surface of the container. In experiments lasting three to four hours, both the amount and rate of uptake was less in the presence of EDTA. There appeared to be no appreciable difference of uptake in light or dark bottles. In the case of *Ceramium,* addition of buffer ("Tris") to minimize the pH changes resulting from photosynthesis indicated pH does not effect the uptake.

In an experiment using *Fucus vesiculosus* it was found that the radioactivity in the medium decreased at a rate less than that in a blank containing no algae. It is speculated that the *Fucus* may release a chelating substance into the medium. (Partially supported by contracts with the A.E.C. and O.N.R.)

Nuclear division in Holosticha lacazei Maupas. Reuben Torch.

Holosticha lacazei, a relatively obscure, multinucleate, free-living, hypotrichous, marine ciliate, was collected on June 23 at Woods Hole and has been cultured in the laboratory for two months.

Nuclear division was studied in animals fixed in Champy's fluid, followed by bleaching in either 3% hydrogen peroxide or dioxan, and stained by the Feulgen reaction or with Heidenhain's hematoxylin. Champy's fixative was used since the animals dissolved in all fixatives except those containing osmium tetroxide.

The number of macronuclei in non-dividing individuals varies from 32 to 71, with most individuals having 40–50. The spherical macronuclei measure 4–8 μ and contain a central

chromatin mass surrounded by numerous small, achromatic spheres. The homogeneous Feulgen-positive spherical micronuclei measure 2 μ and number from 5–13 in non-dividing individuals. There is no apparent correlation between the numbers of macronuclei and micronuclei.

Prior to nuclear division, the macronuclei fuse into an elongate, intensely Feulgen-positive mass in the center of the cell along the longitudinal axis. Concurrently, the daughter adoral membranellae are formed and the future division plane is demarcated. The micronuclei cluster around the fused macronucleus and proceed to divide. Micronuclear division is not synchronized, since no more than half the micronuclei divide at one time. Division of the micronuclei was not observed at any other stage in the life cycle. The micronuclei are distributed more or less equally among the daughters, but in one exceptional case, one daughter contained 6 micronuclei while its sister contained 15.

The macronuclear mass divides approximately in half, one product going to each daughter. Macronuclear divisions continue until each daughter contains 20–30 macronuclei, whereupon the daughters separate. The earlier macronuclear divisions are generally synchronized, but later divisions are sporadic. After separation of the daughters, many of the macronuclei continue to divide. Macronuclear reorganization was not observed at any stage in the life cycle.

Ammonia in Golfingia gouldii and ambient air. DAVID M. TRAVIS.

This work was begun with determination of normal levels of ammonia in ambient air and in coelomic fluid of *Goldfingia gouldii*, in order to devise approaches to a study of effects of increased environmental ammonia gas concentration on metabolic processes. For analysis of air, a large volume, greater than 400 liters, was passed through a cotton filter and two acid traps in series, in order to collect the ammonia. Coelomic fluid was analyzed directly and by use of a trichloracetic acid filtrate prepared immediately after withdrawal from the body cavity. Ammonia was determined by microdiffusion and colorimetric measurement of the reaction with ninhydrin-hydrindantin reagent.

Results were as follows: (1) The ammonia fraction in ambient air was 1.74×10^{-8} (the mean of 14 determinations ranging from 0.55 to 3.47×10^{-8}). (2) Total ammonia of coelomic fluid, using trichloracetic acid filtrate, was 230 micromoles per liter (the mean of 18 determinations, ranging from 115 to 560 micromoles per liter). (3) Direct determinations of ammonia on coelomic fluid at timed intervals after fluid was drawn from the animal showed a rising curve, the flat portion of which was used for extrapolation back to zero time. Values obtained by this extrapolation were about 25% lower than those obtained on the same specimen using trichloracetic acid filtrates.

The results of this work have led to the following approaches to further study of ammonia in physiologic processes: (1) large volumes of ammonia gas may be prepared, analyzed, and used in experimental situations; (2) serial samples of 1 ml. of coelomic fluid may be determined for ammonia in triplicate; (3) release of ammonia into the closed space, containing the animal and suitable reagents, of a respiratory flask may allow precise measurement of metabolic processes.

Respiratory changes induced in Golfingia gouldii by alteration in environmental carbon dioxide. DAVID M. TRAVIS.

Respiration of *Golfingia gouldii* at environmental carbon dioxide concentrations below and above approximately normal levels was studied in order to learn whether this gas exerts effects on the rate of oxygen consumption in the whole animal. For essentially zero carbon dioxide in surrounding air, a volumetric respirometer was used in which carbon dioxide was removed by alkali. For normally small and larger concentrations of environmental carbon dioxide, a syringe method was adopted which allowed measurement of changes in oxygen concentration of a known volume of gas from which oxygen consumption could be calculated. Actual oxygen concentrations, kept between 19.7 and 20%, changed less than 0.5% during periods of one hour.

At zero carbon dioxide tension, oxygen was consumed by the animal at a rate of 33 microliters/hour and gram of wet weight to the 2/3 power ($W^{2/3}$) (mean value for 13 observations on 4 worms, ranging from 20–53). At low concentrations of 0.36–0.99% carbon dioxide,

obtained by allowing equilibration for one hour with room air, the rate was 45% lower, or 15 microliters/$W^{2/3}$-hour (mean of 9 observations on 6 worms, range 9–19). In simultaneous experiments using pairs of worms crossed alternately between low concentrations of 0.49, 0.91 and 0.48% carbon dioxide and higher concentrations, the oxygen consumption was reduced by 27, 35 and 70% when the concentration was raised by 1.0, 3.5 and 8.0% above the lower levels. Oxygen concentrations were approximately the same at the two levels.

From these results it is suggested that carbon dioxide depresses aerobic respiration in *Golfingia gouldii* in approximately physiologic as well as higher pharmacologic concentrations, and, indeed, appears to accelerate respiration when removed from the environment. Studies of mechanisms involved and of the actual role carbon dioxide may play in natural surroundings are indicated.

Studies of cannibal giant Blepharisma undulans. N. TULCHIN AND H. I. HIRSHFIELD.

Previous studies have shown that selected feeding will induce formation of cannibal giant types of the pink, heterotrichous, fresh-water ciliate, *Blepharisma*. The ingestion of smaller blepharisma by the giant forms occurs readily. The fate of the ingested forms can be easily followed in the food vacuole. The digestion of the ingested forms and egestion of non-absorbed substances occurs within twelve hours.

Feulgen and methyl green-pyronin preparations show the DNA and RNA of the cannibal and of the ingested forms within the food vacuole. During digestion, the DNA in the food vacuole is progressively hydrolyzed, as shown by decreasing affinity for methyl green and Feulgen stains. However, pyroninophilia of the mass within the vacuole fluctuates during digestion. RNA-ase and perchloric acid treatments indicate that RNA may persist or accumulate in the vacuole during digestion and that some is present in the egested residues.

The cannibal giant forms have appreciably larger amounts of macronuclear DNA than bacteria-fed controls. The large quantities of DNA in the food vacuoles of the cannibals may thus contribute to the increased polyploidy of the cannibal giant forms.

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Differential staining of cytoplasmic inclusions in eggs of Pectinaria. KENYON S. TWEEDELL.

Constituents of the egg cytoplasm were studied in the germinal vesicle stage, during germinal vesicle breakdown and in the primary oocyte. Inclusions of living eggs were observed before and after centrifugation, using vital fluorochromes, other specific vital dyes and dark-field microscopy. In addition, cytochemical studies were made on fixed eggs before and after centrifugation.

Besides germinal vesicle, nucleolus, chromosomes and spindle, many highly motile inclusions were observed vitally. The latter included yolk, mitochondria, astral granules, germinal vesicle substance, oil droplets and chromidia. Additional stationary, peripherally located granules were seen. By specific cytochemical staining reactions (vital and non-vital) these inclusions could be further differentiated.

Of the vital fluorochromes utilized, acriflavine, thioflavine and acridine orange gave polychromatic staining. Others dyed specific particulates. The fluorescent cell components include cell and nuclear membranes, nucleus, nucleolus, yolk, mitochondria and other unidentified granules.

Two of the vital dyes, toluidine blue and nile blue sulphate, stain several cell components in the germinal vesicle stage. These include nucleolus, peripheral cell granules, yolk and a heavy zone of granules surrounding the germinal vesicle. Both dyes stain the yolk particles blue and show metachromasia with other granules. After centrifugation the metachromatic granules become stratified into separate bands. With toluidine blue, a thin red-violet band of particles occurs just beneath a large cap of unstained granules and droplets at the centripetal pole. When nile blue sulphate is applied, a different red-violet band of small particles stratifies out, just centripetal to the yolk. The astral granules, which become associated with the asters of the first maturation spindle, are derived from this latter band.

Reduction and delay of cleavage in Chaetopterus eggs by acridine orange. K. S. TWEEDELL AND C. D. WATTERS.

Recently, acridine orange was shown to have an inhibitory action on mitosis in *Arbacia* (Rustad, 1959). Additional effects on fertilization and early cleavages were observed when acridine orange was utilized as a vital fluorochrome to follow early development in *Chaetopterus*. The staining period, concentrations of A.O. in sea water, choice of gametes and time of application all affect the number of eggs cleaved.

Acridine orange was applied in concentrations of 10^{-3} (15 minutes), 10^{-4} (30 minutes) and 10^{-5} (60 minutes) in each experiment. When sperm were stained prior to fertilization, the percentage of eggs reaching first cleavage increased (72 to 81%) as the stain intensity decreased. Previously stained eggs in the same concentrations showed an increase in cleavage from 50 to 60% as stain concentration decreased. When both sperm and eggs were dyed prior to fertilization, the percentage of eggs cleaved did not vary significantly from eggs stained alone. Average cleavages in controls for all groups remained constant between 80 and 82%. This suggests that the inhibitory effect on cleavage was principally mediated through the egg.

When eggs and sperm were fertilized in 10^{-3} A.O. and removed after 15 minutes, cleavages were sharply (10%) curtailed. In 10^{-4} A.O., 27% of the eggs cleaved but in 10^{-5} A.O. cleavage recovered (84%). This suggests the factors here either act during fertilization or affect some phase of the cleavage process.

Application of the threshold concentration (10^{-5}) to the egg alone also caused a slight delay (3 minutes) in the first cleavage. When sperm or sperm and eggs are dyed prior to fertilization, an identical delay of 10 minutes occurs. This suggests a sperm-induced cleavage delay. In each case, the rate of development remained constant with the controls. Eggs and sperm fertilized in the stain (then removed) showed no delay in first cleavage, but the rate of succeeding cleavages decreased.

Phosphagen and nucleotides of Limulus muscle. G. W. DE VILLAFRANCA, J. E. ROSS AND A. M. MORGAN.

Horseshoe crab muscle was extracted with trichloracetic acid and precipitated as the alcohol-insoluble barium salt. It was hydrolyzed and the free base chromatographed with arginine, taurocyamine, glycocyamine and creatine standards. The average R_f values in 3 solvents (propanol-ammonia-water, butanol-acetic acid-water, and butanol-ethanol-water) were: arginine, 0.43, 0.14 and 0.07, respectively, and closest to the values found for the *Limulus* base of 0.43, 0.13 and 0.07. The amount of phosphagen, arginine phosphate, was determined by colorimetric assay of the total arginine and the free arginine, the difference being due to bound arginine and assumed to be arginine phosphate. The average values for 6 different animals were 6.26 mM of arginine and 3.10 mM of bound arginine per 100 grams of muscle, wet weight. It is of some interest that the values were always greater than zero, and relatively constant, once it was realized that the crabs must be used fresh. The amount of AP probably decreases once the animal has been removed from its natural habitat; it apparently decreases rapidly and to zero in the running sea water tables.

The nucleotide composition was determined by ion-exchange chromatography (Dowex in the formate cycle) on both barium salts obtained by TCA extraction and, later, on neutralized perchloric acid extracts. In the former case, ATP was the major component with an average of about 100 μM per 100 grams of muscle, fresh weight. ATP was recovered from perchloric acid extracts in concentrations ranging from 163 μM to 338 μM, with an average of 265 μM per 100 grams of muscle. Traces of ADP (43 μM), AMP (26 μM) and other, as yet, unidentified, minor nucleotides were also found.

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Oxygen consumption in Uca pugnax: seasonal variations. H. MARGUERITE WEBB.

O_2-consumption of the fiddler crab, *Uca pugnax*, was recorded continuously from July 25, 1959, through October 6 and for six semi-monthly periods between October 11, 1959, and February 18, 1960; a further lunar period was recorded from June 25 to July 23, 1960. The

mean hourly rate of O_2-consumption was found to be highest during the months of July and August, and to be lowest (about 55% of summer values) during the period from November to February. No gross changes in the form of the *diurnal* rhythm were observed during the period of the investigation nor were there any consistent variations in the amplitude, which ranged from 28% to 43% of the mean hourly rate. When the data were analyzed for *lunar-day* rhythms it was seen that from July 25 through October 25 the rhythm exhibited a symmetrically bimodal form with maxima at lunar zenith and nadir. During the period from November 19 through January 23, although the lunar-day rhythm was less regular than for the earlier periods, it was clearly uni-modal with a maximum about an hour after lunar zenith. During the period from February 2 to 18 the lunar rhythm resumed its bimodal character, but with minima at lunar zenith and nadir. It thus appeared to be inverted with respect to the "summer" rhythm. Like the diurnal rhythm, the lunar rhythm exhibited no systematic changes in percentage amplitude throughout the period studied. During the period from June 25 to July 23, 1960, the lunar rhythm was found to have the "summer" form and phase relations—bimodal, with maxima at lunar zenith and nadir.

This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-122803.

Effects of environmental changes on indefinitely prolonged action potentials of lobster muscle fibers. R. WERMAN, J. P. REUBEN AND H. GRUNDFEST.

Prolonged spikes evoked in muscle fibers treated with a combination of procaine and some other agent (Reuben and Grundfest, this issue) were affected very little by drastic changes in the environment, made during the plateau of the response. The stability of the membrane potential and conductance during the plateau were particularly marked with the responses that had a high membrane conductance (Reuben, Werman and Grundfest, this issue). These spikes were not affected by replacement of all the ions in the bathing medium with sucrose or urea; by replacement of sodium with alkali earth or organic cations; by 20-fold increase in potassium; by substitution of chloride with various anions; or by maximal activation of the inhibitory synapses with γ-aminobutyric acid (GABA).

The spikes with lower-conductance plateaus could be affected to some degree. Bathing the fibers in sucrose or urea induced oscillations, between the plateau and more negative values. Substitution of SCN ions for chloride, which hyperpolarized and increased the membrane resistance of resting muscle fibers, abolished the spikes. Occasionally, the addition of GABA also abolished the spikes.

These findings suggest that in its "upper stable state" the membrane does not behave as a simple electrode for ionic changes. This is particularly the case for conditions in which the "upper stable state" is associated with high membrane conductance.

Relative effectiveness of inhibitory membrane in different fibers of a lobster muscle. R. WERMAN, J. P. REUBEN AND H. GRUNDFEST.

During its maximum activity, the inhibitory membrane of the fibers operates as a high conductance Cl^--electrode, tending to set the membrane at the Cl^--equilibrium potential. The degree to which the synaptic activity prevents changes in the membrane potential that are normally produced by various means is a measure of the relative area of the synaptic membrane in different fibers.

The inhibitory synapses were maximally activated with GABA, in Ringer's solutions containing various amounts of K^+, or none. In the absence of GABA, the membrane potential changed nearly linearly with log K^+, with a slope of 54 mV/decade change in K^+. In zero K^+ there was hyperpolarization of 10 to 15 mV. When the synaptic membrane was activated by GABA, the membrane potential changed relatively little on removal of K^+ in most fibers and not at all in many. On raising K^+ above 15 meq./l. (the value in *Homarus* saline) the slope of the change was again 54 mV/decade change in K^+, but with a different origin. Removal of K^+ during tetanic activation of the inhibitory synapses also produced less hyperpolarization, but abolition of all hyperpolarization, as with GABA, was not seen.

The variation of the clamping action of inhibitory synaptic activity indicates that the con-

ductance of the synaptic membrane was less in some fibers than in others, presumably because different fibers have different areas of innervated membrane. Block of propagation is readily produced during high frequency activity of the inhibitory axon, and accounts for the lower effectiveness of synaptic clamping as compared with that of GABA. The identical slopes of the depolarization curves in high K^+, with and without GABA, reflect the increased conductance of depolarized membrane, and they provide further evidence that the activity of the inhibitory membrane does not involve K^+-conductance.

In situ measurements of the community metabolism of Quohog Pond. GEORGE C. WHITELY, JR.

In situ estimates of oxygen production in Quohog Pond, West Falmouth, were obtained by means of a polarographic oxygen electrode during September, 1959. In July, 1960, the water column at one station was analyzed by Winkler technique for dissolved oxygen every three hours for a duration of 24 hours. Rates of respiration, diffusion, and total photosynthesis were computed graphically for both the September and July data.

Total photosynthesis in a water column one square meter in area was 7 grams oxygen/m^2/day or 2.9 grams carbon/m^2/day for September, 1959; 24 grams oxygen/m^2/day or 10 grams carbon/m^2/day for July, 1960.

During June, 1960, the Marine Ecology Course class study of Quohog Pond determined primary production of the phytoplankton *in vitro* (light and dark bottle method) to be 0.5 gram carbon; benthic algae by the same method averaged 10 grams carbon. Low value for the phytoplankton suggests that bottle measurements alone may have some of the same disadvantages in Quohog Pond as have been found elsewhere; *i.e.*, shallow Texas bays, where such measurements do not take into account the metabolism of benthic organisms.

Production of viable races of Paramecium caudatum after micronuclear elimination with x-rays. RALPH WICHTERMAN.

X-irradiation has been shown to affect micronuclear number in several species of ciliate Protozoa. As a result, there is a diminution of the number of micronuclei and, with reasonably high or repeated dosages, their complete disappearance.

Specimens from clonal cultures of opposite mating types of *Paramecium caudatum* (9 and 10 of variety 5) were treated as follows: 200 counted paramecia were placed in each of four Nylon syringes (2-ml.) and irradiated simultaneously with dosages of 150, 200, or 250 kr, all of which were below the LD 50 value. Immediately after irradiation, some specimens were examined and the remainder expressed from the syringes into flasks of lettuce medium which contained *Aerobacter aerogenes* as the food source. Upon regaining reproductive ability, progeny of x-rayed survivors were then harvested, placed again in syringes and irradiated as before, while others were killed and stained for further cytological study. In this manner seven, approximately evenly spaced irradiation exposures were given over a 52-day period, to bring the cumulative clonal dosage to 1100 kr.

When specimens of opposite mating type from cultures which had received the cumulative clonal dosage of 1100 kr regained reproductive ability and later were mixed, mating and conjugation followed.

Paramecium caudatum has but one compact micronucleus, measuring approximately 8 μ. Repeated and successive x-irradiation of paramecia and their progeny in the manner reported here results in a gradual disappearance of the micronucleus or the production of a "ghost-like" condition of this structure as revealed in stained preparations, whereas the micronucleus from unirradiated controls stains intensely. At the highest cumulative clonal dosage, dividing specimens show either no micronuclear spindle (since such paramecia are now amicronucleate), or else a dividing "ghost-like" micronuclear spindle in contrast to the conspicuous, dividing micronucleus of the unirradiated controls. At least in appearance, the structure of the macronucleus appears little affected by the irradiation.

Part of a project aided by a grant from the Committee on Research, Temple University, and a contract between the Office of Naval Research, Department of the Navy and Temple University (NR 104-475).

The effect of LSD 25 on heart rate in Cistenides. CHARLES G. WILBER.

In previous work, it was shown that the pulsation of the dorsal blood vessel in the trumpet worm is temperature-dependent. The rate response to temperature may be described as $y = 3.5\ x - 49$, where y is beats per minute and x is degrees Centigrade. Approximately 50 worms were immersed in different concentrations of lysergic acid diethylamide (LSD 25) in sea water at 22° C. The effect of this treatment on pulsation of the dorsal vessel was recorded. By 30 minutes after immersion in 20 micrograms LSD 25 per ml. sea water, the rate of pulsation of the dorsal vessel was increased to 130% of control. In more concentrated solutions, the worms became flaccid, extended, and the dorsal vessel ceased to beat. In sea water containing 10 micrograms per ml. LSD 25, there was no change in rate, or possibly a depression to 10% of control. By way of comparison, worms immersed for 30 minutes in 10 micrograms per ml. atropine sulfate in sea water showed a decrease to 59% of control rate. In 20 micrograms per ml. the rate increased to 120% of control. After two hours in the above solutions, the rates were essentially the same as those indicated above. The LSD 25 was kindly supplied by the Sandoz Pharmaceutical Company.

Supported in part by National Science Foundation Grant 4005.

Cardiovascular and respiratory reflexes in elasmobranchs. CHARLES G. WILBER AND FREDERICK N. SUDAK.

During a simulated dive, certain mammals, birds and reptiles show a profound bradycardia with accompanying changes in blood pressure. Fish are said to show similar changes if removed from the water. To ascertain in some detail the nature of such responses in elasmobranchs, we used specimens of *Squalus acanthias* and of *Mustelus canis*. The unanesthetized fish were immobilized in a plastic trough. Fresh sea water was run into the mouth through a plastic hose. Blood pressure was recorded directly from the ventral aorta with a pressure transducer. Heart rate was read from the pressure record. Respiration was recorded through electrodes placed directly into the branchial muscles. Control records were made with full water flow. The water was then shut off; heart rate, blood pressure and respiratory rate were monitored for several minutes. Within 6 to 20 seconds after water flow was stopped, the following physiological responses (expressed as change to per cent of control) occurred: *Squalus* heart rate 45%, respiratory rate 51%, pulse pressure 144%, mean pressure 82%; *Mustelus* heart rate 42%, respiratory rate 19%, pulse pressure 214%, mean pressure 90%. The heart rate response in both species was abolished by atropinization of the fish. Atropine abolished the pulse pressure response in *Squalus* but not in *Mustelus*. The observed bradycardia is apparently mediated through the vagus nerve. Atropinization did not modify the respiratory response in either species. The small changes in mean blood pressure, despite relatively large decreases in heart rate, after interruption of water flow, do not confirm the view that this response serves to protect delicate gill structures.

Supported in part by research grants MY-3235 and H-3563 from the National Institutes of Health.

Some effects of lysergic acid diethylamide on circulation in elasmobranchs. CHARLES G. WILBER AND FREDERICK N. SUDAK.

Lysergic acid diethylamide (LSD 25), a psychotogenic agent in man, is known to provoke bradycardia in mammals by central vagal stimulation. The agent induces a drop in blood pressure as a result of depression of the vasomotor center, despite the vasoconstrictive action which the drug exhibits in perfused blood vessels or in spinal animals. We made some experiments with LSD 25 on *Mustelus canis*. Blood pressure and heart rate were measured before and at different times after various doses of LSD 25 were administered via the ventral aorta. Doses from 0.01 to 1.3 micromol per kg. body weight were given. After all doses there was a rise in systolic, diastolic, mean, and pulse pressures. Heart rate, which decreased by 40% at a dose of 1.3 micromol per kg., showed no decrease at doses less than 0.01 micromol. The rise in mean pressure amounted to 15% of control value at a dose of 0.01 micromol; 45% at 0.06 micromol; 81% at 0.56 micromol. Various methods of plotting dose against blood pressure

response were tried. At the moment, the data seem to fall along a curve in the most orderly fashion if dose in micromol per kg. is plotted against the reciprocal of the per cent change in blood pressure. The results are now being processed through a computer to arrive at the best mathematical expression of the dose-response curve. The LSD 25 was kindly supplied by Sandoz Pharmaceutical Company.

Supported in part by research grants MY-3235 and H-3563 from the National Institutes of Health.

An autoradiographic study of the regenerating limb in the adult newt, Triturus viridescens. RUTH T. WILF.

The source of cells for the blastema of the regenerating limb of the adult newt, *Triturus viridescens,* has been investigated, using autoradiography. In this two-year study the radioisotopes used were tritiated thymidine (principally), and C^{14} isotopes of glycine, leucine, and methionine. With tritiated thymidine, we assume takeup of radioisotope only in DNA-synthesizing, premitotic cells. In limbs with high epidermal cell labeling compared to control non-regenerating limbs, or with positive internal cells, regenerative activity of these cells is assumed.

With tritiated thymidine the approaches were: (1) time course regeneration study from third to twenty-eighth day post-amputation; injection and fixation on same day to give immediate picture of new cell formation; (2) newts injected from third to twenty-first day; fixation on subsequent days to study cellular divisions and migrations; (3) following the discovery that prior to the fifth day, only epidermal cells were labeled, tritiated thymidine was injected four hours pre-amputation or three days post-amputation, to test a possible epidermal cell contribution to blastema.

It was found that internal mesodermal components contribute to the blastema. From the fifth day many dedifferentiating muscle nuclei, muscle sheath nuclei, and some subcutaneous nuclei are synthesizing DNA. The periosteum is strikingly labeled. Schwann cells are generally negative. These conditions persist until after the blastema is well formed. The labeled dedifferentiated mesodermal cells divide and contribute extensively to the blastema. The blastema itself has a high rate of synthetic activity, which falls off as the blastema lengthens.

The epidermis is usually particularly heavily labeled in a band at the level of the amputation. These cells migrate distally continuously until about the eighteenth day, to form the bulk of the apical cap of epidermis, although some DNA synthesis is almost always taking place across the top of the cap. There is no evidence that epidermal cells contribute to the blastema.

Heat tolerance and thermoregulation in the fiddler crab, Uca pugilator. JERREL L. WILKENS.

Fiddler crabs often feed in their natural habitat during the hottest part of a day in direct sunlight. The following experiments were undertaken to determine the upper extremes of temperature fiddler crabs can tolerate, and, under these conditions, if the effects of evaporation and radiation would be of any survival value. To determine environmental heat tolerances, freshly collected animals were subjected to temperature increases for one-hour periods over sea water and in dry air. In saturated air, 50% of the population died at 40.5° C. and 100% at 42° C. In dry air, 50% died at 45.5° C. and 100% at 47° C. The crabs, therefore, have a thermal tolerance of as much as 10 to 15° above average daily temperature (about 30° C.) for the Woods Hole area. However, temperatures approaching 45° C. were occasionally recorded on the surface of the sand; therefore, some thermoregulatory mechanism would be an advantage.

Through the use of thermocouples, internal temperatures of light-adapted and of dark-adapted specimens were determined during exposure to infra-red radiation in still air. The body temperatures approached equilibrium about 6° C. below ambient air temperatures in light crabs and 3° C. below air temperatures in dark crabs, for periods up to 15 minutes. These plateaus are due primarily to radiation and evaporation. To determine the effects of evaporation alone, crabs were subjected to slowly moving air at 37.5° C. Body temperatures leveled

off at 33.5° C. in dry air, at 35.5° C. in air at 50% relative humidity, and at 37.5° C. in saturated air. The rate of evaporation of water was essentially proportional to increasing temperature.

This work was undertaken as part of the student training program of the Marine Ecology Course, and was partially supported by the Graduate School of Tulane University.

Experiments with ovulation induced in vitro by means of steroids in frogs and marine fishes. PAUL A. WRIGHT.

Certain androgenic, progestational, and cortical steroids have potent ovulatory capacity on isolated lobes of frog ovary, *Rana pipiens*, when added in amounts ranging from 2 to 0.001 mg. in 50 ml. of Ringer's solution and in the absence of pituitary factors. Several compounds (*e.g.*, progesterone, corticosterone, cortisone-21-alcohol) induced responses equaling or exceeding those brought about by optimal concentrations of frog pituitary extract. These same classes of steroids in moderate dosage enhanced ovarian response to pituitary factors *in vitro*, but overdoses often inhibited ovulatory responses. Estrone, estradiol, and diethylstilbestrol were incapable of inducing ovulation by themselves, and inhibited pituitary-induced ovulation, diethylstilbestrol > estradiol > estrone. Interestingly, these same estrogens did not inhibit ovulation *in vitro* induced by progesterone, testosterone, or hydrocortisone, but instead augmented the ovarian response to these ovulatory steroids.

Attempts to induce ovulation in excised ovaries of marine fishes (*Lophius, Fundulus, Tautoga*) by means of either steroids or pituitary extracts were not successful. A few eggs were released from a ripe ovary of *Fundulus majalis* with both progesterone and *Fundulus* pituitary extract, but controls in Ringer's solution also released ova spontaneously.

Aided by research grant A-2986 from the National Institutes of Health, USPHS. Thanks are due to Dr. Preston L. Perlman of Schering Corporation for supplying a variety of steroids for these experiments.

Metabolism of three species of the gastropod genus Littorina in and out of water. ROGER G. ZIEG.

Results of the experiments conducted on three intertidal snails of the Woods Hole region demonstrated that the respiration rate of these animals varied significantly when in water and when exposed. The three forms considered are dispersed, in more or less definite zones, on the shore. *Littorina palliata* is primarily in an aquatic habitat among the sea-weeds, *Littorina rudis* is confined to the upper regions of the shore exposed during low tide, *Littorina littorea* appears to be intermediate, being neither restricted to the exposed nor the submerged zones.

Rate of oxygen consumption was determined by Winkler method for measurements "in water," and a manometric method for measurements "out of water." Snails were collected at random and respiration measurements obtained for periods not exceeding three hours.

Results obtained by these methods, from about 500 snails of each species, indicate that: *L. palliata* gave mean values of 1.60 ml. O_2/gm./hr. in air and 1.01 ml. O_2/gm./hr. in water ($P < .001$). Results indicate that *L. littorea* consumes .54 ml. O_2/gm./hr. in air and .34 ml. O_2/gm./hr. in water ($P < .001$). On the other hand, *L. rudis* has lower respiration rates in air than in water, mean values of .59 ml. O_2/gm./hr. in air and .91 ml. O_2/gm./hr. in water ($P = .01$) being obtained.

The metabolic rates measured in air and in water tend to reflect the differential distribution of the three gastropods in the intertidal environment.

This work was carried out as a part of the student training program of the Marine Ecology Course.

A study of the nucleic acids of the dogfish cornea. SEYMOUR ZIGMAN AND WILLIAM P. AREND.

Our objective is to determine whether nucleic acids, due to their influence over protein synthesis, have a function in the maintenance of tissue transparency. Initial efforts were

directed toward the development of a procedure for measuring the nucleic acid phosphorus content of smooth dogfish (*Mustelus canis*) corneas.

A modification of the Schmidt and Thannhauser method for the fractionation of acid-soluble, lipid, ribonucleic acid (RNA), deoxyribonucleic (DNA), and protein phosphorus components of the cornea was employed. All fractions contained appreciable amounts of phosphorus, both in the epithelium and stroma, except the protein phosphorus, which was nearly absent. As was expected, the highly cellular epithelium was far richer in RNA and DNA phosphorus than the very fibrous stroma.

These results led us to investigate the uptake of P^{32} by the compounds mentioned. Several incubations of whole corneas or of separated epithelia and stromas were attempted, incubating with elasmobranch Ringer's solutions containing 1 μc. of P^{32} for two and four hours. Since little incorporation of radioactivity into the nucleic acids was observed, it seems that more suitable systems must be sought.

Further work was carried out to develop a method for the quantitative analysis of the nucleotides of corneal RNA by paper electrophoresis. Although complete separation of 50 μg. of pure nucleotide solutions or of yeast RNA hydrolysates could be accomplished, corneal nucleic acid hydrolysates have not as yet given clear-cut separations of cytidylic, adenylic, guanylic and uridylic acids.

Future studies will include similar experiments on such transparent tissues as teleost corneas, avian nictitating membranes and squid integument.

Isolation of mitotic apparatus from pressurized Arbacia eggs. ARTHUR M. ZIMMERMAN AND DOUGLAS MARSLAND.

Mitotic apparatus (spindle-aster-chromosome complex) were isolated from the eggs of *Arbacia punctulata* immediately subsequent to a short exposure to high (up to 10,000 lbs./in.^{2}) pressure, applied at metaphase of first cleavage. The isolation procedure consisted of selective solubilization with digitonin after treatment with cold 35% ethanol.

In general it was found that high pressures had marked disorganizing effects upon the structure of the mitotic apparatus. However, the disorientation of the spindle-aster configuration decreased progressively as the magnitude and duration of the treatment were decreased.

A pressure of 10,000 lbs./in.^{2} applied for only one minute yielded spindle-aster remnants completely devoid of any detectable linear or radial structure, although the centriole areas and chromosomes were clearly visible. The chromosomes were clumped very irregularly near the center of the spindle area. Often the spindle area was abnormally short and sometimes curved.

In contrast to the foregoing result, seemingly normal spindle-aster complexes were recovered from eggs exposed to 4000 lbs./in.^{2} for one minute. The fibrillar structure of the spindle and asters was clear, the chromosomes and centriole areas appeared normal, and judging from the large number of anaphases found, movement of the chromosomes had not been abolished.

At intermediate pressure (8000 lbs./in.^{2}) a longer treatment (5 minutes) was required to approximate the effects previously described, and a still longer treatment (10 minutes) produced even greater disorganization.

Presumably the observed disorganization of the spindle and asters results from a pressure-induced solation of these intracellular gel structures. Moreover, the evidence suggests that the spindle is slightly more susceptible than the asters to such solation.

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Uptake of adenosine nucleotides in Arbacia eggs. ARTHUR M. ZIMMERMAN AND SELMA B. ZIMMERMAN.

Studies were conducted to determine the mode of penetration of adenosine triphosphate (ATP) and related adenine nucleotides into the eggs of *Arbacia punctulata*. Arbacia eggs

were immersed into C^{14} labeled adenosine triphosphate, adenosine diphosphate and adenosine monophosphate, and subsequently prepared for autoradiographic analysis.

Experiments with unfertilized eggs immersed into $C^{14}ATP$ (at 20–22° C.) for varying periods of time show that effective uptake was obtained after 3–4 hours immersion at relatively low ATP concentrations (5×10^{-4} to 10^{-8} *M*). Shorter immersion times of 60 and 120 minutes resulted in negligible amounts of C^{14} uptake. The uptake of labeled ATP in fertilized eggs was greater than that in unfertilized eggs. When eggs were placed into 5×10^{-4} *M* $C^{14}ATP$ (0.12 μc/ml.) 5 minutes after insemination, an appreciable uptake of radioactivity was observed at the following metaphase. Qualitatively, the amount of uptake after 30–35 minutes incubation in the nucleotides was comparable to that found in unfertilized eggs incubated for a duration of 3–4 hours.

Preliminary observations of ATP-treated eggs (fertilized and unfertilized), centrifuged just prior to fixation, indicate that the radioactivity within the eggs is associated with the mitochondrial and yolk zones. Further studies are being conducted in order to determine the mechanism of uptake of ATP, ADP, and AMP.

This work was done with the technical assistance of Miss Patricia Rodgers.

Supported by grant G-6416 from the National Science Foundation.

A report of hermaphroditism in Arbacia punctulata. ARTHUR M. ZIMMERMAN, SELMA B. ZIMMERMAN AND ETHEL BROWNE HARVEY.

Hermaphroditism is not frequently found in the sea urchin, *Arbacia punctulata*. To date there have been only seven reported instances in this species. The hermaphrodite described in this report was given to the investigators at the Marine Biological Laboratory, Woods Hole, by Dr. E. E. Palincsar on the 17th of June, 1960.

Viable eggs and sperm were obtained simultaneously from the hermaphrodite upon electrical stimulation. Four of the gonads yielded eggs and the fifth gave sperm. Controlled electrical stimulation made it possible to collect unfertilized eggs. These eggs could then be fertilized with sperm from the same animal. Furthermore, cross-fertilization was possible, using sperm from another animal and eggs from the hermaphrodite. The unfertilized eggs averaged 71 μ in diameter. The fertilized eggs in the two- and four-cell stage measured 78 μ (without fertilization membrane). This is within the range of normal eggs.

Developmental studies under carefully controlled temperature conditions showed that the duration of time from insemination to 50% first cleavage furrows was 58–60 minutes at 20° C. This is similar to that found in normal control eggs. Both the cross-fertilized and the self-fertilized eggs were normal with respect to their morphology and rates of development to the pluteus stage.

The work was supported in part by grant G-6416 from the National Science Foundation and grant RG-7157 from the Division of General Medical Sciences, U. S. Public Health Service.

LALOR FELLOWSHIP REPORTS

On the natural inhibitors of glucuronosyl transferase present in serum of pregnant women. ROBERT M. DOWBEN.

The enzyme glucuronosyl transferase catalyzes the transfer of the glucuronide moiety from uridine diphosphoglucuronic acid (UDPGA) to a large number of hormones, metabolic products and drugs which are excreted as glucuronide conjugates. Previous studies disclosed the presence of inhibitors of this enzyme in the serum of pregnant women; one inhibitor was tentatively identified as pregnanediol-3α,20α, a metabolite of progesterone.

Ten liters of pooled serum obtained from pregnant women in the last trimester of pregnancy were acidified with sulfuric acid to pH 1.6 and extracted in batches with ethyl acetate for 12 hours by means of a continuous liquid-liquid extractor. All of the inhibitory activity of the serum was extracted by this means. The combined extracts were chromatographed on an alumina column, using a benzene-ethanol developer. A number of fractions possessing inhibitory activity have been obtained and assessed by measuring the decreased conjugation of

phenolphthalein by a rat liver microsomal system in the presence of UPDGA. Aliquots of several of the more polar fractions lost most of their inhibitory activity after hydrolysis with β-glucuronidase. Similarly, the inhibitory activity of the pooled serum was greatly diminished by treatment with β-glucuronidase, indicating that some of the inhibitors may be glucuronides. The chemical identification of the various fractions is to be pursued.

The membrane surrounding milk fat globules. ROBERT M. DOWBEN AND DELBERT E. PHILPOTT.

The fat globules in cream were observed in the electron microscope. Fat in milk occurs as rather uniform globules about 3–4 microns in diameter. These fat globules do not coalesce to form butter unless the cream is churned, sonicated or frozen. Heat, treatment with fat solvents such as ethyl ether, and dilution with distilled water are much less effective in disrupting the fat globules. Calcium salts appear to stabilize the fat globules.

The cream of raw cow's milk was separated by centrifugation and washed three times with five volumes of 0.25 *M* sucrose containing 0.01 *M* $CaCl_2$. The third washings contained no detectable protein by the biuret reaction. Analysis of the washed cream revealed about 2.4 mg. protein/g. cream. The washed cream was examined in the electron microscope, using neutral mammalian isotonic osmic acid solution or neutral isotonic osmic acid dichromate solution for fixation.

The protein surrounding the fat globules appears to be a true membrane rather than adsorbed protein. The globules could be lysed by freezing and thawing, giving rise to discoid ghosts which resembled in many ways erythrocyte ghosts.

Serotonin (5-hydroxytryptamine) in the male reproductive tract of the spiny dogfish. THADDEUS MANN.

The male copulatory organs or "claspers" of the spiny dogfish, *Squalus acanthias*, by means of which semen is transferred from the male to the female, are associated with accessory abdominal organs known as "clasper siphons" which were believed for a long time to act merely as a reservoir of sea-water. In the course of an investigation on the function of these organs it was observed that the fluid obtained by flushing the siphon sacs with water gives a strong reaction with diazo reagents. Fluids obtained in this manner from five males were pooled, deproteinized, and the protein-free extract, representing a dry weight of 175 mg., subjected to further analyses. These have shown that the diazo-reactive substance is identical with serotonin (5-hydroxytryptamine). On the basis of optical activity measured at 275 mμ, the serotonin content of the protein-free extract was found to represent 6.2% of the dry weight. Determination based on absorption measurement of the purple-colored reaction product with 1-nitroso-2-naphthol gave a closely similar result, namely, 6.4%. Paper chromatograms sprayed with either diazobenzenesulfonic acid, para-dimethylaminobenzaldehyde, or ninhydrine, yielded spots with the same R_F value and color as pure serotonin.

Serotonin is known to possess the ability to stimulate uterine contractions. The participation of the fluid derived from the clasper siphons in the formation of fish seminal plasma, and the abundance of serotonin in that fluid suggest that, in the spiny dogfish at any rate, serotonin may play a role in the process of reproduction, either by influencing contractions of the female reproductive tract, thus facilitating sperm passage and fertilization, or by affecting the mechanism of copulation and ejaculation in the male.

Lipid-soluble sialic acid containing material in Arbacia eggs. L. WARREN AND R. HATHAWAY.

An Arbacia egg contains 3.8×10^{-4} μgm. bound sialic acid which can be completely released by hydrolysis for 2 hours at 80° C. in 0.1 N H_2SO_4. None of the sialic acid is normally free (*i.e.*, reactive in the thiobarbituric acid assay before hydrolysis) and there is none in the jelly coat.

At least two forms of sialic acid-containing substances are present in the egg, one of

which is soluble in organic solvents. Approximately 25% of the total sialic acid can be readily extracted into n-butanol from a sea urchin egg homegenate. The lipid sialic acid complex is soluble in chloroform-methanol (2:1) and in ether. The ether solubility suggests that the carboxyl group of the sialic acid residue may be blocked. Free sialic acids (N-acetylneuraminic acid and N-glycolylneuraminic acid) are insoluble in ether.

The sialic acid is released from the complex in one hour in 0.1 N H_2SO_4 at 80°. It is also released enzymatically by sialidase of *Vibrio cholerae*. The freed sialic acid has been purified by chromatography on a column of Dowex-1-acetate and has the following properties: It demonstrates the same color reactions as authentic material in the orcinol, Ehrlich, and thiobarbituric acid tests. It co-chromatographs on paper in two different solvent systems with N-glycolylneuraminic acid and separates completely from N-acetylneuraminic acid.

Other components of the lipid-soluble sialic acid-containing complex of Arbacia eggs described here are not known. The evidence obtained to date suggests that it differs from the gangliosides and other mucolipoproteins of animal brain.

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Sialic acid in semen of Arbacia punctulata. L. Warren, R. Hathaway and J. G. Flaks.

Sialic acid is present in bound form in both the seminal plasma and sperm of Arbacia. Sialic acid appears to be present in at least two forms in seminal plasma: a readily hydrolyzable type (67%) which is cleaved within 30 minutes at 80° in 0.1 N H_2SO_4, and another form (33%) which takes four hours to hydrolyze under the same conditions. In one experiment 50% of seminal plasma sialic acid was enzymatically removed by the sialidase of *Vibrio cholerae*.

There are approximately 9.5×10^{-4} μgm. sialic acid in an Arbacia sperm. When sea water-washed sperm are treated with fertilizin, a soluble non-dialyzable sialic acid-containing material is removed from the sperm. The reaction appears to be complete within one minute and there is no further solubilization of sperm material unless more fertilizin is added. In one experiment four successive fertilizin treatments of a preparation of sperm led to the removal of 20% (12.0, 3.6, 3.2, 1.2%) of the sperm sialic acid. Heated fertilizin (80°, 5 minutes) was also active. Sialic acid substances in sperm may also be removed by detergents, such as sodium lauryl sulfate or cetyltrimethylammonium chloride. Four successive extractions of sperm by a 7.5×10^{-4} *M* sea water solution of sodium lauryl sulfate led to the removal of 82% (55.0, 18.8, 9.2, 5.9) of the sperm sialic acid, and to a loss of 25% of sperm dry weight. Approximately 12% of the material removed from the sperm could be accounted for as sialic acid. The sperm were still motile after the second extraction. Sperm also releases sialic acid-containing substances when suspended in distilled water and when incubated at 45° or higher, for 5 minutes.

Incubation of the solubilized sperm material at 80° for four hours in 0.1 N H_2SO_4 is required to cleave the sialic acid residue from the nondialyzable part. Only 5% of the sialic acid can be removed enzymatically. The sialic acid obtained by acid hydrolysis has been purified on a column of Dowex-1-acetate and gives the thiobarbituric acid, orcinol, and Ehrlich tests for sialic acid. On paper it co-chromatographed with N-glycolylneuraminic acid. The selective removal of materials from sperm by fertiiizin appears to take place in a rapid non-enzymatic manner. The biological activities of the substances removed from sperm are described in an adjoining abstract.

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